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FACULTY OF SCIENCES

Department of Biology

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Nutrient removal from horticultural waste water by natural and assembled communities of benthic algae

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Front cover: A graphic abstract of Chapter 4.

Back cover: A brief introduction of this thesis.

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Abbreviations

ATS	Algal Turf Scrubber
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
FA	Fatty acid
FAME	Fatty acid methyl esters
MUFA	Mono-unsaturated fatty acid
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acid
RAD	Global solar irradiance
SFA	Saturated fatty acid
TFA	Total fatty acid
UFA	Unsaturated fatty acid

Nomenclature

F_0	Dark fluorescence yield
NO_3^--N	Nitrate nitrogen (mg L^{-1})
PO_4^{3-}-P	Phosphate phosphorus (mg L^{-1})
R	Removal rate (mg N, P L^{-1} day^{-1})
RE	Removal efficiency (%)
TN	Total nitrogen (mg N L^{-1})
TP	Total phosphorus (mg P L^{-1})
μ	Specific growth rate (d^{-1})

Chapter 1

Introduction and thesis outline

1. General introduction

The exponential human population increase and the rapid industrialization have produced tremendous volume of domestic, agricultural and industrial wastewater and have greatly increased the input of primary nutrients including nitrogen and phosphorus as well as other pollutants into natural water bodies since the mid-20th century (Abdel-Raouf et al., 2012; Boelee et al., 2014; Posadas et al., 2015). A survey of the International Lake Environment Committee during 1988 and 1993 showed that 48% of lakes and reservoirs in North America were eutrophic; in Asia and the Pacific, it was 54%; in Europe, 53%; in South America, 41%; and in Africa, 28% (Cai et al., 2013). Worldwide, eutrophication and oxygen depletion of freshwater aquatic ecosystems have resulted in the loss of key species and ecosystem functions and the deterioration of surface water quality (Renuka et al., 2015). As a consequence, combating eutrophication and pollution of aquatic resources is increasingly being implemented in water policy regulations.

World issues regarding depletion of resources and global warming call for a wastewater treatment with efficient nutrient recovery especially phosphorus and decreasing greenhouse gases emissions (Shilton et al., 2012; Van Den Hende, 2014). Therefore, conventional wastewater treatment systems solely focusing on pollutants removal are facing increasing scrutiny. As shown in Fig. 1.1, the traditional biological nitrogen and phosphorus removal techniques including activated sludge treatment and denitrification pond generate a great volume of sludge waste, release nitrogen to the atmosphere and use additive chemicals (Craggs et al., 1996; Renuka et al., 2015; Renuka et al., 2013). The higher aquatic macrophytes systems, such as reed bed sewage systems and constructed wetlands, require large area of land and are low in nutrient removal capacity (Kern & Idler, 1999; Renuka et al., 2015; Vrhovšek et al., 1996).

A considerable number of studies have demonstrated the potential of algae for nitrogen and phosphorus removal since 1957 (Abdel-Raouf et al., 2012; Boelee et al., 2011; Chinnasamy et al., 2014; de-Bashan & Bashan, 2010; Oswald & Gotaas, 1957). Algae offer the advantages of having high growth rates and being capable of assimilating nitrogen and phosphorus from wastewater with low operational cost, less land requirement, no secondary pollution, efficient recovery of nitrogen and phosphorus, no requirement of organic carbon and no CO_2 emission (Abdel-Raouf et al., 2012; Aslan & Kapdan, 2006; Boelee et al., 2011; Renuka et al., 2013). In addition to reclaiming nutrient from wastewater, the algae-based wastewater treatment system can also produce valuable algal biomass, such as protein, biofertilizer, fatty acids, biofuel and other high-

value extracts (Fig. 1.1) (Arbib et al., 2013; Chinnasamy et al., 2014; Mulbry et al., 2010; Renuka et al., 2015). In the last half-century, algae have been widely employed in treating secondary effluent, agricultural, domestic, piggery and dairy wastewaters (Abdel-Raouf et al., 2012; Mulbry et al., 2010; Zamalloa et al., 2013; Zhu et al., 2013).

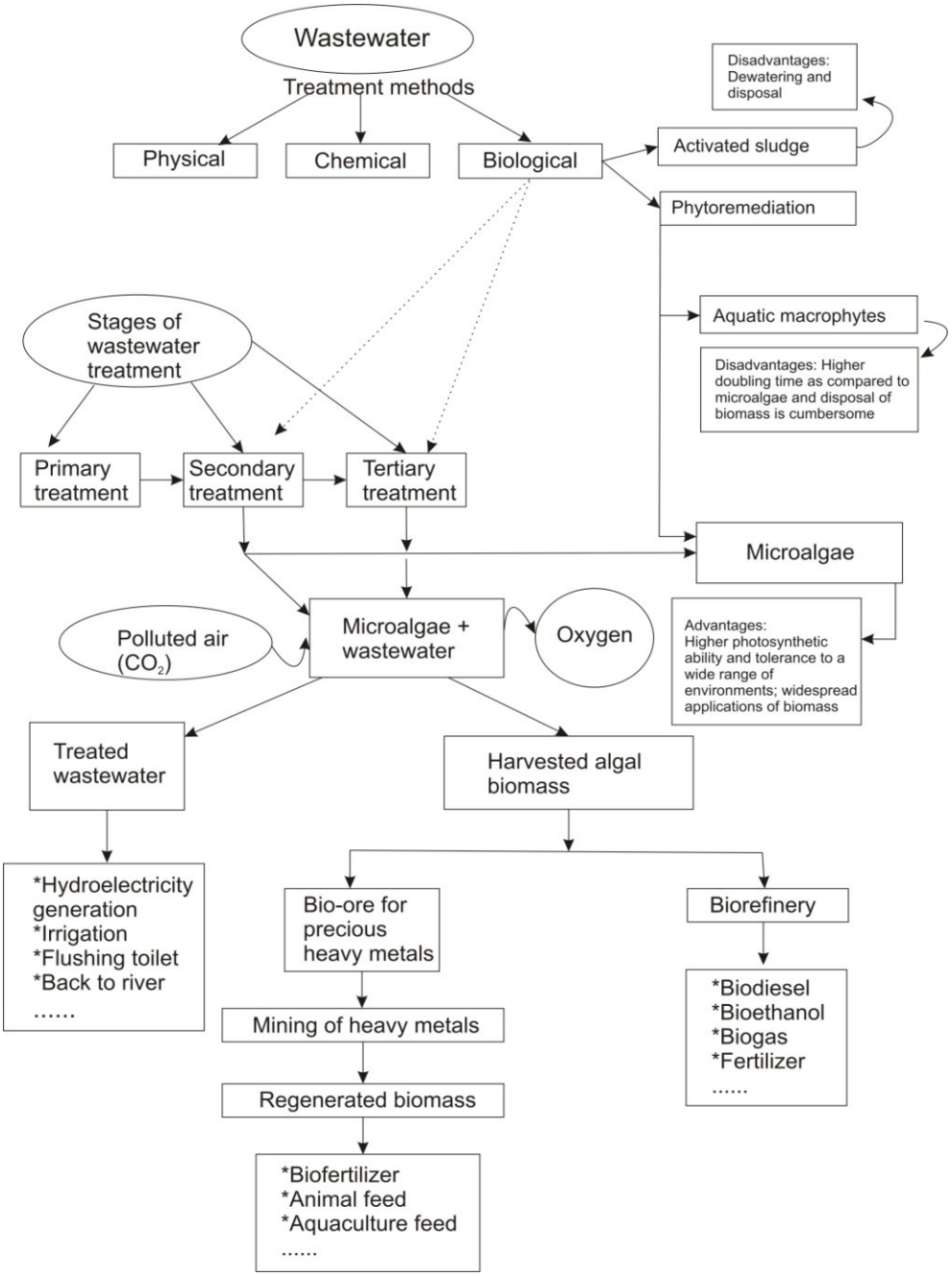

Fig. 1.1 Schematic representation of wastewater treatment using microalgae: overview of advantages and applications (Adapted from Renuka et al., 2015 and Zhu, 2015)

However, the harvest and separation of planktonic algal biomass from water are time and energy consuming and remain the major bottlenecks in large-scale applications (Abdel-Raouf et al., 2012; Vandamme et al., 2013). Furthermore, the species-specific

variations in nutrient uptake, adaptation to different wastewaters, tolerance of environmental changes (e.g. temperature and solar irradiance), and predation by invertebrate grazers should be taken into consideration as well (Abdel-Raouf et al., 2012; Cai et al., 2013; Renuka et al., 2015).

This chapter gives an overview of the most relevant literatures that form the background of this work. First, it introduces the characteristics of microalgae and microalgae-based mechanisms and technologies in wastewater treatment. Second, it gives summary of the applications of benthic filamentous algae based systems in nutrient removal from wastewater. Third, it gives introductions of benthic algae-based bioreactor Algal Turf Scrubber in reclaiming nutrient from wastewater. Finally, the objectives of this work and the outline of its structure are defined.

2. Microalgae

2.1 What are microalgae?

The organisms generally regarded as algae are those with chlorophyll *a* and a thallus, but not differentiated into roots, stem and leaves (Richmond, 2004; Vymazal, 1995). Therefore, the term "microalgae" refers to the microscopic algae. Microalgae are found all over the world, mainly distributed in the waters, but also on the surface of all type of soils (Vandamme, 2013). Microalgae comprise a very diverse group ranging from unicellular to multicellular and from sphere, ellipsoid, elliptic prism to cylinder in shape (Hillebrand et al., 1999; Richmond, 2004; Vymazal, 1995). The photosynthetic mechanisms of microalgae are similar to that of higher plants, but microalgae are able to assimilate nutrient efficiently from their aquatic environment and have great potentials to efficiently transform light into biomass (Vandamme, 2013).

2.2 Biochemical composition of microalgae

To date, microalgae including *Chlorella*, *Dunaliella*, *Haematococcus* and *Spirulina*, have received increasing attentions in biotechnology research with regard to their utilizations for human and animal nutrition, and high value added biochemical products, such as astaxanthin, β-carotene, linoleic acid and γ-linolenic acid (Babuskin et al., 2014; Cole et al., 2015; Wan et al., 2014; Zhu, 2015). Many microalgal strains can also accumulate a great amount of lipids under nitrogen limited conditions and be used as biofuel feedstock (Griffiths et al., 2012; Han et al., 2013; Li et al., 2011).

Furthermore, cultivation conditions can have significant influences on algal biochemical composition (Griffiths et al., 2012; Richmond, 2004). Nitrogen limitation is a key factor triggering the reduction of protein and the accumulation of lipids and fatty acids of many microalgae strains and the effects are species-specific (Bona et al., 2014; Cha et al., 2011). For example, Griffiths et al. (2012) reported that nitrogen limitation greatly increased the lipids content of *Chlorella vulgaris* and *Scenedesmus* sp. by 2.5-4.6 times, while it had no effect on the lipids content of *Spirulina platensis*. Light intensity and light/dark cycles have significant effects on algal growth and biochemical composition and low light has been shown to favor the formation of PUFAs for many algal species (Hu et al., 2008; Sharma et al., 2012). Napolitano (1994) reported that the C18:3ω3 content of the filamentous alga *Cladophora* sp. was sensitive to changes in irradiance and its proportion of TFAs increased significantly from 12.2 to 25.8% when the light intensity changed from 1500 to 30 μmol photons m^{-2} s^{-1}. Moreover, the biochemical composition can vary greatly in different growth phases. Liang et al. (2006) reported that the increasing culture age caused an increase of C16:1ω7 and C18:1ω9 in *Phaeodactylum tricornutum* and C16:0 in *Chaetoceros muelleri*.

3. Microalgae in wastewater treatment

The use of algae in wastewater treatment has been investigated for nearly 60 years with one of the first reports by Oswald and Gotaas (1957). Biological wastewater treatment with microalgae is gaining wide acceptances because of their capabilities of converting solar energy into valuable biomass and taking up nutrient such as nitrogen and phosphorus which may cause eutrophication when released to natural water bodies (Abdel-Raouf et al., 2012; Cai et al., 2013). Currently increasing interest has been developed worldwide such as in Australia, USA, China and Europe (Abdel-Raouf et al., 2012; Boelee et al., 2011; Li et al., 2010a).

3.1 N and P removal via algae

The principal forms of nitrogen and phosphorus in wastewater are NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}. Microalgae have a high capacity of assimilating inorganic nitrogen and phosphorus, and they can grow in mass culture in outdoor bioreactors, thus microalgal cultures offer an elegant solution to wastewater treatment (Roberts et al., 2013).

Similarly to other organisms, nitrogen is a critical nutrient in algal growth and organic nitrogen exists in a variety of biological substances in algal cells such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP) and ge-

netic materials (RNA, DNA) (Cai et al., 2013). The organic nitrogen in algal cells is assimilated from inorganic sources including NO_3^-, NO_2^- and NH_4^+. In addition, some cyanobacteria can fix N_2 from atmosphere (Havens et al., 2003).

As shown in Fig. 1.2, translocation of the inorganic nitrogen occurs across the plasma membrane, followed by the reduction of oxidized nitrogen and the incorporation of ammonium into amino acids. Nitrate reductase uses the reduced form of nicotinamide adenine dinucleotide (NADH) to transfer two electrons, resulting in the conversion of nitrate into nitrite with the assistance of nitrate reductase. Nitrite can then be converted into ammonium by nitrite reductase and ferredoxin (Fd). Thus, all forms of inorganic nitrogen are ultimately converted into ammonium. Then, ammonium can be incorporated into the amino acid glutamine through glutamine synthase by using glutamate (Glu) and ATP (Cai et al., 2013).

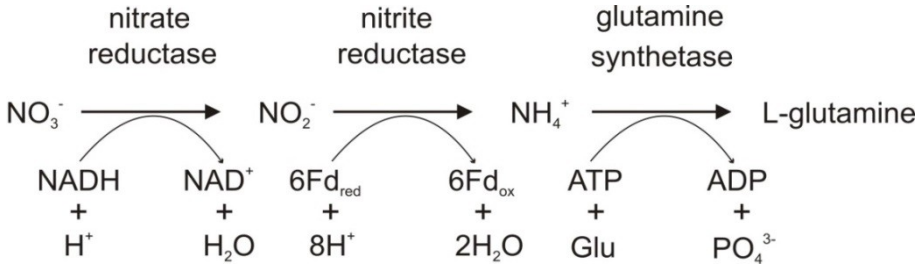

Fig. 1.2 Schematic of the assimilation of inorganic nitrogen (Adapted from Cai et al., 2013)

From the nitrogen assimilation process of algal cells (Fig. 1.2), it can be concluded that ammonium is the preferred form by algae because no redox reaction is needed in its assimilation and nitrate consumption will not happen until ammonium is completely consumed (Arbib et al., 2013), but excessive ammonium will have a repressive effect on algal growth. It has been reported that the ammonium tolerance of different algae species varies from 25 to 1000 mmol NH_4^+-N L^{-1} (Cai et al., 2013). Except for the assimilation by algal cells, ammonium can also be removed by stripping, through which the volatilization of ammonium happens at increased pH or temperature (Aslan & Kapdan, 2006). Although nitrate is not the preferred nitrogen form in the assimilation, it is highly oxidized and a stable and predominant form of nitrogen in wastewater. Thus, nitrate can be an important nitrogen source for microalgae at the presence of nitrate reductase and be assimilated from wastewater by algae.

Similarly to nitrogen, phosphorus also plays a key role in the metabolism of algae and can be found in nucleic acids, lipids, proteins, and the intermediates of carbohydrate metabolism (Cai et al., 2013). Inorganic phosphates are important in algae cell

growth and metabolism. During algae metabolism, phosphorus, especially in the form of $H_2PO_4^-$ and HPO_4^{2-}, is incorporated into organic compounds through the generation of ATP from ADP, accompanied by a form of energy input across the plasma membrane of the algal cell (Formula 1.1). Besides inorganic phosphorus, some algae species can also use organic phosphorus for growth.

$$2NADP + 3H_2O + 2ADP + 2P_i \rightarrow 2NADPH_2 + 3ATP + O_2 \quad (1.1)$$

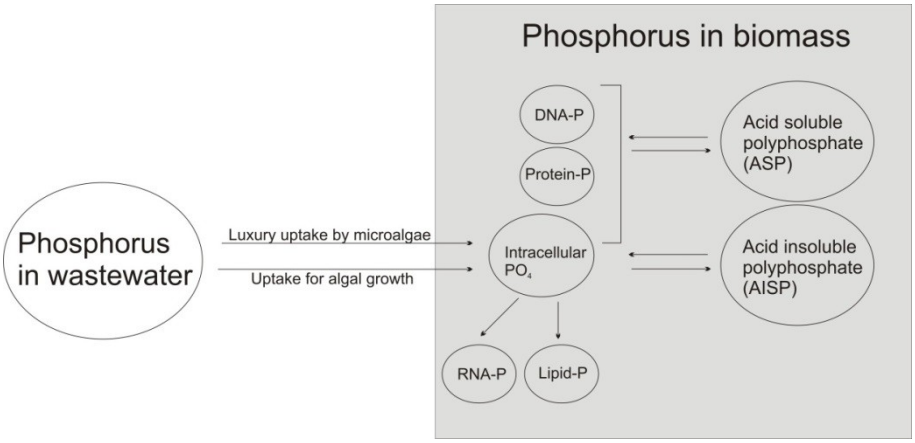

Fig. 1.3 Summary of phosphorus uptake by microalgae from wastewater (Adapted from Powell, 2009)

The phosphorus in algal cells can be divided into two forms: acid-soluble polyphosphate (ASP) and acid-insoluble polyphosphate (AISP) (Powell et al., 2009). Acid-soluble polyphosphate is used for metabolism and production of DNA and proteins, while acid-insoluble polyphosphate is a form of phosphorus storage that can be utilized by the cell when the external phosphorus concentration becomes limited for algal growth (Fig. 1.3) (Powell et al., 2009). Phosphorus removal from wastewater mainly contains two ways: one is the uptake into algal cell, and the other one is through precipitation with metal ions caused by pH elevation via algal photosynthesis (Craggs et al., 1996; de-Bashan & Bashan, 2004). For phosphorus uptake, it contains the basic uptake for algal growth and luxury uptake (Powell, 2009). Luxury uptake of phosphorus in microalgae means the microalgae take up more phosphorus than required for growth without a prior starvation stage (Powell et al., 2009) and it can be influenced by temperature, phosphate concentration and light (Powell et al., 2008). Furthermore, phosphorus can also be removed from wastewater through surface adsorption via the formation of hydrogen bonds with the polysaccharides secreted by algae or bacteria in a biofilm (Li et al., 2013; Lu et al., 2014; Sheng et al., 2010).

3.2 Factors affecting algal growth and nutrient removal

In order for a successful, continuous and cost-efficient wastewater treatment system, various environmental and operational factors must be taken into account. These factors can influence algal photosynthesis, biomass productivity as well as nutrient removal efficiency and biochemical composition of the resultant biomass (Griffiths et al., 2012; Markou & Georgakakis, 2011). Generally, the most important factors include nutrient availability, irradiance, temperature, cultural cell density and interactions with other microorganisms (Table 1.1).

Table 1.1 Factors relevant to algal growth and nutrient removal in bioreactors for wastewater treatment (Adey et al., 2011; Bosma et al., 2014; Kesaano & Sims, 2014; Larsdotter, 2006; Markou & Georgakakis, 2011)

Abiotic factors, physical and chemical	Light (quality, quantity)
	Temperature
	Nutrient concentration
	N/P ratio
	O_2, CO_2
	pH
	Salinity
	Toxic chemicals
Biotic factors	Algal composition
	Pathogens (bacteria, fungi, viruses)
	Predation by zooplankton
	Competition between species
Operational factors	Mixing
	Dilution rate
	Depth
	Addition of bicarbonate
	Harvesting frequency

3.2.1 Light

Microalgae are photosynthetic organisms by using light energy to extract protons and electrons from H_2O to reduce CO_2 to form organic molecules (Formula 1.2).

$$6H_2O + 6CO_2 + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (1.2)$$

At low irradiance, the rate of photosynthesis depends linearly on light intensity (Fig. 1.4). With increasing light intensity, photosynthesis becomes less and less efficient. Finally, it reaches the light-saturated value where enzymatic reactions become rate lim-

iting. Under prolonged supra-optimal irradiance, photosynthetic rates will decline from the light-saturated value as photoinhibition of photosynthesis (Richmond, 2004). In addition to the photosynthesis rate, irradiance can also have significant effect on the biochemical composition of algal cells including pigment and fatty acid (Babuskin et al., 2014; McLarnon-Riches et al., 1998).

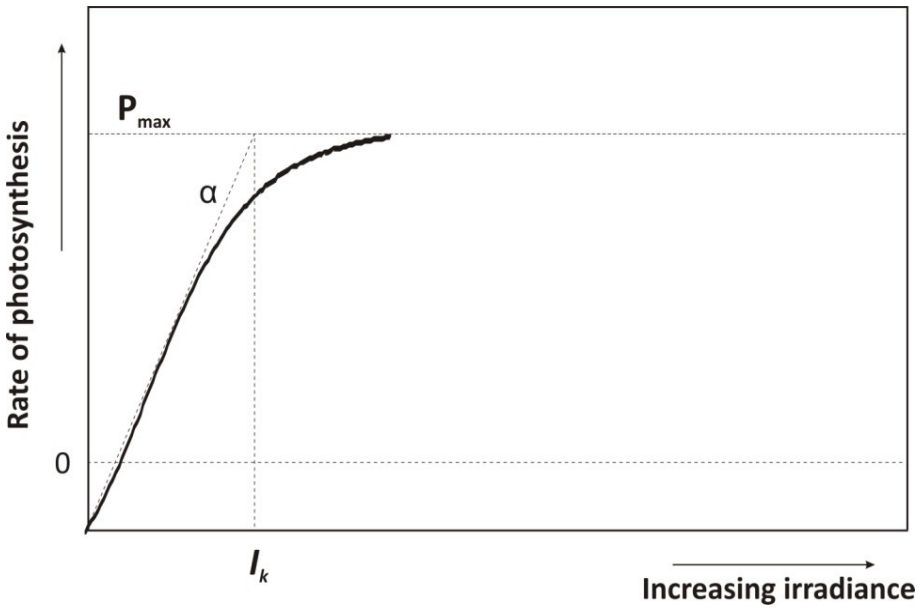

Fig. 1.4 A schematic representation of photosynthetic light-response curves. The initial slope of the curve (α) is the maximum light utilization efficiency. The intersection between the maximum rate of photosynthesis P_{max} and α is the light saturation (optimum) irradiance. At supra-optimum irradiance, photosynthesis declines, which is commonly called down-regulation or photoinhibition (Adapted from Richmond, 2004)

3.2.2 Temperature

The effect of temperature on algal growth can be described by the Arrhenius Law where microalgal growth increases with increasing temperature up to an optimum growth temperature, then microalgal growth rapidly decreases above the optimum growth temperature until lethal temperature is reached (Boelee et al., 2014). The optimum growth temperature is genera and strain dependent. For instance, optimal temperature for *Scenedesmus obliquus* and *Anabaena variabilis* is 25-30 °C and 35 °C respectively, while for *Spirulina* it is about 30-38 °C (Breuer et al., 2013; Markou & Georgakakis, 2011). Moreover, temperature can have effects on the biochemical composition of algal cells, such as fatty acid and triacylglycerol (Breuer et al., 2013; McLarnon-Riches et al., 1998).

3.2.3 Nutrient

Carbon is an essential nutrient for algal cultivation and can be taken up from inorganic and organic forms. Inorganic carbon is utilized through the CO_2 concentrating mechanism and algae have the ability to utilize both CO_2 and HCO_3^- as an inorganic carbon source (Markou & Georgakakis, 2011). Many studies have attempted adding CO_2 to promote algal growth and nutrient removal (Craggs et al., 2012). Moreover, the heterotrophic algal species can use organic carbon, for instance *Chlorella* can use glucose as carbon source (Bhatnagar et al., 2011; Perez-Garcia et al., 2011).

Nitrogen is another important nutrient for the production of microalgal biomass. The nitrogen content of the biomass can range from 1% to more than 10% and is dependent upon the amount, the availability and the type of nitrogen sources (Li et al., 2010b). Nitrogen can be utilized as NO_3^-, NO_2^- or NH_4^+ and N_2 as well. The nitrogen availability of the wastewater is highly related to other nutrient uptake such as phosphorus, which is assimilated through active transport and depends on the transport protein (Beuckels et al., 2015; Perini & Bracken, 2014).

Phosphorus is another essential macro-nutrient for microalgae growth. Although algal growth doesn't need large amounts of phosphorus (less than 1% of dry weight), low phosphorus concentration is related to low cell densities (Markou & Georgakakis, 2011). However, many algae are able to take up excess phosphorus and store it as polyphosphate as an internal source, which can be used for prolonged culture in phosphorus deficient media (Markou & Georgakakis, 2011; Powell, 2009).

In addition to the absolute concentrations of nitrogen and phosphorus, the N/P ratio of wastewater varies greatly from 1 to over 35 (in weight) and can directly influence algal growth, nutrient removal and biochemical composition (Arbib et al., 2013; Krishnan et al., 2008; Li et al., 2010a; Zhu et al., 2013). For example, Molina et al. (1991) reported that the specific growth rate of *Tetraselmis* sp. increased significantly from 0.04 h^{-1} to 0.06 h^{-1} following the change of N/P ratio from 0.5 to 5. A study of Li et al. (2010a) documented that nitrogen removal efficiency of *Scenedesmus* sp. decreased significantly with N/P ratio higher than 15:1. Moreover, protein content of algal biomass is related to the nitrogen availability in the medium, so the wastewater of high N/P ratio can be a suitable source for protein production (Arbib et al., 2013). While in wastewater of low N/P ratio, which produced a nitrogen limiting condition, a great amount of lipid can be accumulated in algal cells (Arbib et al., 2013; Li et al., 2010a).

3.2.4 Algal community

It is well-known that different algal groups differ greatly in their growth rate, biochemical composition and thus their C: N: P stoichiometry may vary between phyla but also between related species (Burkhardt et al., 1999; Geider & La Roche, 2002; Ho et al., 2003; Quigg et al., 2003). For example, Ho et al. (2003) documented that under identical culture conditions, two green algae *Dunaliella tertiolecta* and *Nannochloris atomus* had an N/P mole ratio of 38 and 25 respectively, while the N/P ratio of four diatom species *Ditylum brightwellii*, *Thalassiosira weissflogii*, *Nitzschia brevirostris* and *Thalassiosira eccentric* varied between 5.4 and 13.6. As a consequence of the variation in algal stoichiometry, the nutrient requirement and uptake rate are species-specific. In the study of Pedersen and Borum (1997), several macroalgae including *Chaetomorpha*, *Cladophora*, *Codium* and *Ulva* had an ammonium and nitrate uptake rate ranging from 81 to 240 μmol NH_4^+-N g^{-1} dry weight h^{-1} and 9 to 30 μmol NO_3^--N g^{-1} dry weight h^{-1} respectively. In a study of Wallentinus (1984), several algae including *Cladophora glomerata*, *Enteromorpha ahlneriana* and *Stigeoclonium tenue*, had a high nitrogen or phosphorus uptake rate of 5.7 mg NH_4^+-N g^{-1} dry weight h^{-1} and 4.2 mg PO_4^{3-}-P g^{-1} dry weight h^{-1}, while for some other species the nutrient uptake rates were only 0.2 mg NH_4^+-N g^{-1} dry weight h^{-1} and 0.02 mg PO_4^{3-}-P g^{-1} dry weight h^{-1}. These contrasting nutrient uptake rates stress the difference in nutrient requirement and thus nutrient removal capability between different algal species. Therefore, it is crucial to select the appropriate species for improving nutrient removal efficiency from wastewater.

3.2.5 Interaction with other microorganisms

Outdoor cultures of microalgae in wastewater suffer from contaminations of other microorganisms, such as bacteria, fungi, yeasts and other microalgae genera, the metabolites of which may inhibit the growth or predate on the cultivated microalgae (Markou & Georgakakis, 2011). Although the purpose of wastewater treatment is to efficiently remove pollutants from wastewater, biomass with specific characteristics is preferred from the energy balance viewpoint, such as a high content of protein or unsaturated fatty acid (Cole et al., 2015; Mulbry et al., 2010).

3.2.5.1 Predation by invertebrate grazers

Microalgae are usually palatable for small zooplankton and susceptible to grazing by *Chironomids*, *Gastropods*, Trichopteran larvae, Ephemeropteran larvae, and

crustaceans (Guo et al., 2014; Kesaano & Sims, 2014; Van Den Hende, 2014). The presence of grazers can significantly reduce the algae densities to low levels within just a few days, especially in summer (Hillebrand & Kahlert, 2001; Kesaano & Sims, 2014). The parasitic fungi, such as *Chytridium* and *Aphelidium*, can also cause unexpected crash of mass microalgal cultures (Van Den Hende, 2014). Van den Hende et al. (2014) reported that 25% of the biomass production measurements from their wastewater treating algal ponds were negative due the predation by *Tubifex* sp., which had a population of 10-20 per kg dewatered biomass. To reduce the effects and populations of grazers, Mulbry et al. (2008b) added *Bacillus thuringiensis* larvicide to Algal Turf Scrubber to control the population of chironomid larvae and Guzzon et al. (2008) froze the growth medium at -20 °C for 24 h to remove grazers.

3.2.5.2 Priority effects

The arrival order of species in a developing ecosystem can have long-lasting influences on the community structure, referred to as priority effects (Louette & De Meester, 2007; van Gremberghe et al., 2009). Two mutually non-exclusive mechanisms may be responsible for the priority effects. First, the early-arriving species gain precedence to limiting resources including nutrient, light and space, and had a rapid population growth. Thus the carrying capacity can be reached before the later-arriving species become abundant (Kardol et al., 2013; van Gremberghe et al., 2009). For example, Kardol et al. (2013) reported that under high nutrient supply, the early-arriving plant species grew quickly and took up light and space, which made it hard for the later-arriving species to establish. While under low-nutrient conditions, the early-arriving plant growth was slow and light and space remained available for later-arriving species to establish in the community. Second, early-arriving species can also alter the environment in a favorable or detrimental way for later-arriving species (van Gremberghe et al., 2009). For instance, some filamentous green algae including *Cladophora* and *Spirogyra* can suppress phytoplankton's growth by releasing allelochemicals (Trochine et al., 2011). The shading of plants can inhibit the germination of seeds, but can also facilitate the growth of seedlings in semiarid environments by protecting them against drought (van Gremberghe et al., 2009).

3.2.5.3 Interaction between microalgae and bacteria

In any wastewater cultivated algal ecosystem, the interaction between microalgae and bacteria is inevitable (Van den Hende et al., 2014). As photosynthetic microor-

ganisms, microalgae consume CO_2 and produce O_2, while bacteria take up O_2 and produce CO_2 through degrading organic materials in wastewater (Van Den Hende, 2014). Thus, the interaction between microalgae and bacteria could have positive effects on algal photosynthesis through reducing O_2 dissolved in wastewater and producing CO_2. Furthermore, denitrification is another microbially facilitated process of nitrate reduction by anaerobic bacteria. During denitrification, nitrate is the electron receptor and is reduced to NO_2^-, NO, N_2O and finally N_2 (Formula 1.3) (Strohm et al., 2007).

$$2\ NO_3^- + 10\ e^- + 12\ H^+ \rightarrow N_2 + 6\ H_2O,\ \Delta G_0 = -333\ kJ/mol \quad (1.3)$$

3.3 Alternative algal culture and wastewater treatment systems

Based on the physiological features of microalgae, many open or closed systems have been developed in the mass culture of microalgae as well as in wastewater treatment with a scale of less than 1 m^2 or 1 m^3 to over 1000 m^2 or 1000 m^3, including Algal Disk, Algal Turf Scrubber (ATS), High Rate Algal Pond (HRAP), Rotating Algal Biofilm Reactor (RABR) and Flat Plate and Tubular Photobioreactors (PBRs) (Abe et al., 2008; Adey et al., 2011; Gross et al., 2015a; Gross et al., 2015b; Huang et al., 2015; Johnson & Wen, 2010; Kesaano & Sims, 2014; Liu et al., 2013; Park et al., 2011; Singh & Sharma, 2012).

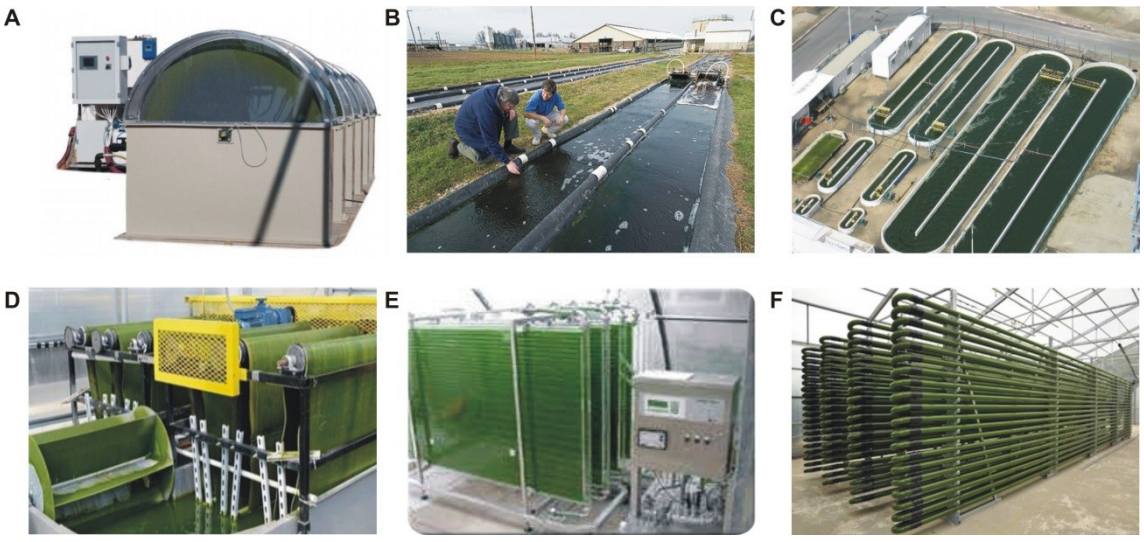

Fig. 1.5 A-F: Photos of Algal Disk, Algal Turf Scrubber, High Rate Algal Pond, Rotating Algal Biofilm Reactor, Flat Plate Photobioreactor and Tubular Photobioreactor

Algal disk (Fig. 1.5A) is a system in which algae can grow both in an aqueous environment and on biocompatible surfaces, allowing for CO_2 absorption from either the gas or liquid phase (www.algadisk.eu). Algal Turf Scrubber (Fig. 1.5B) is an at-

tached cultivation system using benthic algae based periphyton biofilm (Adey et al., 2011). A high rate algal pond (Fig. 1.5C) is usually less than 0.3 m in depth to provide sufficient sunlight for photosynthesis of microalgal cells. The algae culture is mixed and circulated around the pond track by paddlewheels (Cai et al., 2013). The rotating algal biofilm reactor (Fig. 1.5D) is a system in which algal cells grow on the surface of a material rotating between nutrient-rich liquid and CO_2-rich gaseous phase (Gross et al., 2013). Flat plate photobioreactor (Fig. 1.5E) is a kind of photobioreactor with large illuminated specific surface area, short light path, small site area and low energy consumption (Huang et al., 2015). Tubular Photobioreactor (Fig. 1.5F) is a suspended algal cultivation system with large illumination surface area and usually connected with glass or plastic tubes (Michels et al., 2015).

3.4 Biomass product for further utilizations

Algae have the ability of efficiently assimilating dissolved nutrient from wastewater and converting them into protein, essential amino acids, polyunsaturated fatty acids and pigments (Cole et al., 2015; Van Den Hende, 2014). As reported by Becker (2007), the protein content of algal biomass ranged between 6 and 71% of dry weight. Therefore, the wastewater fed microalgae can be used as protein supplements and food additives in animal or aquaculture feed (Renuka et al., 2015).

Phosphorus is a vital element for every plant and animal and it becomes increasingly scarce, thus its recovery from wastewater is of high necessity (Shilton et al., 2012). The algal biomass produced from wastewater treatment is usually rich of phosphorus (1-4%) and can be a viable source of phosphorus fertilizers (Chinnasamy et al., 2014; Mulbry et al., 2005).

Production of algae as a biofuel feedstock has been the subject of research for at least five decades and the high cost is still the bottleneck to compete with other feedstock (Chinnasamy et al., 2014). Thus, the mass cultivation of algae in wastewater becomes a viable option for producing algal biomass at a low cost and simultaneously purifying the wastewater (Chinnasamy et al., 2014). There are already a considerable number of studies on the biofuel production of wastewater-grown algae and the total lipids content can be up to 34% of dry weight (Arbib et al., 2013; Zhou et al., 2011). Therefore, the wastewater treatment coupled with biofuel production can not only remove nutrient from wastewater, but also reduce the cost of biofuel production (Craggs et al., 2012).

3.5 Challenges of microalgae-based wastewater treatment

The commonly studied algae for wastewater treatment are planktonic species, such as *Chlorella*, *Dunaliella* and *Scenedesmus* (Aslan & Kapdan, 2006; Bich et al., 1999; Li et al., 2010a; Zhu et al., 2013), but their harvest is time and energy consuming and accounts for about 20-30% of the total costs of microalgal biomass cultivation and therefore remains a major obstacle for its large-scale application (Barros et al., 2015; Molina Grima et al., 2003). Many studies have tried to reduce the harvesting cost, including the immobilization of algae in polymers (de-Bashan & Bashan, 2010) and harvesting through flocculation (Vandamme et al., 2013), using various kinds of systems and substrate materials, such as rope, cotton sheet, glass and polyvinyl chloride for culturing algae (Borowitzka, 1999; Cai et al., 2013; Gross et al., 2013; Hoffmann, 1998; Johnson & Wen, 2010; Posadas et al., 2013; Schnurr et al., 2013). Furthermore, unicellular algal cells are usually palatable for grazers, which may cause productivity losses or even the crash of mass cultures (Guo et al., 2014; Wang et al., 2014).

4. Benthic filamentous algae and nutrient removal

Recently, research efforts have increasingly focused on non-planktonic algae, with high surface attachment, flotation capacity, or self-aggregation capacity (Olguin et al., 2003; Wang et al., 2013) which can reduce the harvesting cost. Particularly, because of their large cell/colony size and the ability of forming a biofilm attached to the substrate, filamentous algae such as *Cladophora*, *Spirogyra*, and *Tribonema* are attractive because they can be easily harvested by scrapping the biofilm from the substrate (Mulbry & Wilkie, 2001; Olguin et al., 2003; Wang et al., 2013). Based on these characteristics, several systems have been built in the wastewater treatment, such as Algal Turf Scrubber (Adey et al., 2011; Adey et al., 2013), Algal disk (Liu et al., 2013), Rotating Algal Biofilm Reactor (Gross et al., 2013; Johnson & Wen, 2010), Biofilm Membrane Photobioreactor (Gao et al., 2015) and Twin-layer Photobioreactor (Shi et al., 2014). In the small scale Algal Turf Scrubber of Mulbry et al. (2010), which was dominated by the filamentous algae *Enteromorpha* sp., *Lyngbya* sp. and *Spirogyra* sp., the biomass was easily harvested by a wet/dry vacuum followed by dewatering by sieving through a 2 mm nylon netting, and resulting in a 10% solids content of concentrated biomass. In another pilot scale Algal Turf Scrubber predominated by filamentous algae, the biomass was also efficiently harvested by a vacuum nozzle and blower (Craggs et al., 1996).

In addition to the easy harvest, filamentous algae are more resistant to the predations of invertebrate grazers because of their large cell/colony size and thick and indigestible cell wall with high cellulose content (Guo et al., 2014; Wellnitz & Ward, 1998). Those characteristics can benefit for reducing biomass loss by predation and preventing the crash of mass culture (Guo et al., 2014; Wang et al., 2013).

5. Algal Turf Scrubber: a periphyton-based bioreactor

Algal Turf Scrubber (ATS, Fig. 1.6) is an attached cultivation system developed by Adey and coworkers in the 1980s (Adey et al., 1993) based on periphyton biofilm for wastewater treatment. Generally to say, the ATS is a controlled ecosystem by flowing wastewater over an inclined surface which is covered with periphyton biofilms mainly composed of algae, bacteria, protozoa and small multicellular animals (Craggs et al., 1996; Larned, 2010; Mulbry et al., 2008b; Sandefur et al., 2011). So far, ATS has wide applications in treating polluted river water, agricultural wastewater, dairy manure and domestic wastewater, and has been approved to be a simple-constructed and cost-effective wastewater treatment system (Craggs et al., 1996; Mulbry et al., 2010; Mulbry & Wilkie, 2001; Pizarro et al., 2002).

5.1 Composition of ATS

The ATS is an artificial stream designed to promote biological wastewater treatment based on benthic algae community, by driving its photosynthesis to high levels, and harvesting it periodically to remove the metabolites and stimulate further production (Adey et al., 2011; Sandefur et al., 2011). The microbes in the ATS, such as bacteria, fungi and protozoa, can break down organic compounds and excrete them into simpler forms to dissolve in the wastewater (Craggs et al., 1996). The essential elements of the ATS system are a solid support for the growth and harvest of periphyton, wave surge and light (Adey et al., 2011; Craggs et al., 1996). Accordingly, light intensity, temperature, algal species composition, flow rate, nutrient loading and substrate material are the vital factors in this periphyton-based system (Adey et al., 2011; Craggs et al., 1996; Larned, 2010; Mulbry et al., 2010; Mulbry et al., 2008a; Mulbry et al., 2008b; Sandefur et al., 2011).

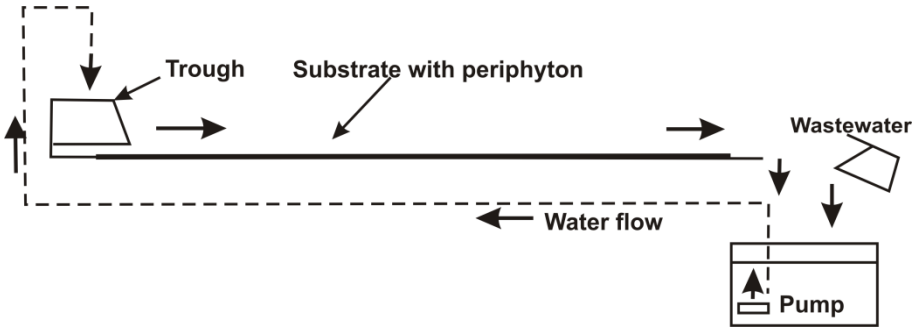

Fig. 1.6 A schematic drawing of the Algal Turf Scrubber with periphyton biofilm (Adapted from Mulbry et al. 2010)

5.2 Factors relevant to ATS

Similarly to the monocultures of microalgae, the irradiance and temperature are also critical factors for the photosynthesis of outdoor-growing benthic algal community and thus the biomass production and nutrient removal rate (Mulbry et al., 2010). As shown in Table 1.2, the annual average biomass production of ATS achieved 5-48 g DW m^{-2} d^{-1} (Craggs et al., 1996; Guzzon et al., 2008; Sandefur et al., 2011) and it varied greatly between the seasons. One research of Craggs et al. (1996) showed that in June and July, the biomass production was around 50-60 g DW m^{-2} day^{-1}, while it decreased to 8-12 g DW m^{-2} day^{-1} in December and January. Mulbry et al. (2010) reported that the average nitrogen and phosphorus removal rates were 250 mg (N) and 45 mg (P) m^{-2} d^{-1} respectively from May to October, then decreased to 16 mg (N) and 3 mg (P) m^{-2} day^{-1} from December to February. In addition to the variation of biomass production and nutrient removal, the algal species composition also showed a great variation from spring to winter (Craggs et al., 1996) with new dominating species of *Microspora*, *Cladophora*, *Ulothrix*, *Stigeoclonium*, *Spirogyra*, *Tribonema* and *Rhizoclonium* appeared in summer and autumn. However, there has been no report on the application of Algal Turf Scrubber under the temperate climate of Western Europe.

Wastewater composition and loading rate are also critical factors in periphyton metabolism, and a high nutrient concentration or loading rate leads to high biomass production and thus nitrogen and phosphorus removal rates and biochemical composition of biomass as well (Adey et al., 2011; Kebede-Westhead et al., 2006; Kebede-Westhead et al., 2003). A laboratory-scale ATS (Pizarro et al., 2002) showed that with NH_4^+-N concentration changing from 5 to 80 mg L^{-1}, NH_4^+-N removal rate increased from 1 to 4 μmol min^{-1} g^{-1} DW, while PO_4^{3-}-P removal rate increased from 0.07 to 0.4 μmol g^{-1}DW min^{-1} when PO_4^{3-}-P changed from 1 to 15 mg L^{-1}. One research of Mulbry

et al. (2008b) showed that mean algal productivity increased from 2.5 g DW m^{-2} d^{-1} at a loading rate of 0.3 g N, 0.05 g P m^{-2} d^{-1}, to 24 g DW m^{-2} d^{-1} at a higher loading rate of 2.5 g N, 0.40 g P m^{-2} d^{-1}. A study of Kebede-Westhead et al. (2003) showed that nitrogen and phosphorus content of biomass increased from 3.6 and 0.6% of DW to 6.9 and 1.1% respectively when the wastewater loading increased from 2 to 9 L m^{-2} d^{-1}.

Table 1.2 Algal biomass production of ATS in literature

Experiment	Maximal biomass production (g DW m^{-2} d^{-1})	Wastewater	Reference
Periphyton biofilm	47.7	River water	Adey et al., 2013
Periphyton biofilm	23.0	Dairy manure	Kebede-Westhead et al., 2003
Periphyton biofilm	35.0	Sewage	Craggs et al., 1996
Periphyton biofilm	5.0	Agricultural drainage	Kangas & Mulbry, 2014
Periphyton biofilm	5.0	Dairy manure	Mulbry & Wilkie, 2001

In an ATS, the algal biofilm mat is periodically harvested to stimulate algal growth and nutrient removal efficiency and simultaneously reduce the predator population and detachment of the periphyton biofilm (Chinnasamy et al., 2014). Therefore, a proper harvest frequency is crucial in maximizing the biomass production and nutrient removal, and a harvest interval of a week in summer and two weeks or longer in winter has been proposed by Craggs et al. (1996) and Adey et al. (2013).

The ATS system promotes algal nutrient uptake by utilizing the surging motion of water, so a high flow rate enhances the mass exchange and thus biomass production. The flow rate determines the ability of water to hold and transport suspended solids (Godillot et al., 2001), drive biological metabolism and chemical reactions, and it also facilitates nutrient uptake by bringing metabolites to reaction sites and carrying away the waste (Craggs et al., 1996). The work of Zippel et al. (2007) showed that under the same indoor light and temperature conditions, the biomass production was significantly higher at a flow rate of 100 L h^{-1} than at 25 L h^{-1}, but Guzzon et al. (2008) concluded that effects of water velocity can be easily masked by the fluctuations of light intensity and temperature under outdoor conditions.

However, benthic filamentous algae grow via attaching to the supporting substrate, the shear stress caused by water flow can break the attachment and filamentous algae have been proved sensitive to shear stress and a low flow rate or water velocity is usually preferred (Biggs & Thomsen, 1995; Larned, 2010). Ahn et al. (2013) and Dodds

(1991) reported that a low flow rate enhanced the dominance of benthic filamentous algae of their periphyton biofilms. Therefore, flow rate or water velocity could be an operation factor in periphyton formation, algal community composition, biomass production and nutrient removal of an ATS.

Furthermore, the propensity of algal strains to attach to the substrate is another major factor to consider in an attached cultivation system (Gross et al., 2015a). Algal cells generally have a negative surface charge and the relative strength of the negative charge determines the hydrophobicity of the cell membranes. In turn, cells that are more hydrophobic tend to be superior at forming biofilms (Gross et al., 2015a; Ozkan & Berberoglu, 2013).

Extracellular polymeric substances (EPS) produced by bacteria in the algal community also play an important role in cell attachment and biofilm formation (Kesaano & Sims, 2014; Sheng et al., 2010), so the bacteria strains capable of producing a large amount of EPS are expected to form biofilm easily and preferred in an ATS.

5.3 Limitations of ATS

As a solar energy driven system under natural conditions, the performance of ATS in wastewater treatment highly depends on the temperature and solar irradiance (Adey et al., 2011). Especially for those operated in temperate regions, winter temperatures are usually below 0 °C and the outdoor algal growth is not possible (Mayr et al., 2015). Similarly, in the regions with overcast and wet weather, light can have limiting effects on the ATS (Kesaano & Sims, 2014). Consequently, the biomass production and nutrient removal performance of ATS often show great fluctuations over different seasons, which make it hard to maintain continuous and stable nutrient removal efficiency (Adey et al., 2013; Craggs et al., 1996; Sandefur et al., 2011).

6. Objectives and outline of this thesis

Benthic filamentous algae are considered as promising organisms to study because of their particular biochemical composition, remarkable capacity of assimilating nitrogen and phosphorus and their capability of attaching to the substrates for cost-efficient harvesting and biomass dewatering. The native microalgal species growing in a certain region are usually more adapted to the local climate and are expected to perform better in growing and nutrient removal from wastewater than the commercially available species. Therefore, four chapters and a general discussion explore the biochemical

composition and nutrient uptake capacity of the natively growing benthic filamentous algae in Belgium under controlled indoor conditions, and the possibility of implementing these algae and their communities through priority effects in treating real horticultural wastewater in outdoor Algal Turf Scrubber with producing valuable biomass.

Chapter 2 Microalgae vary greatly in their growth rate and biochemical composition (e.g. fatty acid, pigment, protein, lipid, etc.) and their responses to growth phase and culture condition changes are species-specific. Four filamentous green algae (*Klebsormidium* spp., *Stigeoclonium* sp. and *Uronema* sp.) were newly isolated from natively growing biofilms in a municipal wastewater treatment plant in Belgium. Accordingly, their growth characteristics, biochemical composition including protein content and fatty acid profile were investigated at different growth phases and under nitrogen-deplete or dark exposure conditions.

Chapter 3 Different algal groups differ greatly in their C: N: P stoichiometry and thus their capacity of taking up nutrient from wastewater. Thus, wastewaters with various nitrogen and phosphorus concentrations and ratios can have direct effects on algal growth and nutrient removal process. Accordingly, in this chapter the effect of varying N/P ratios on algal growth, nutrient uptake and composition of four newly isolated benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and *Pseudanabaena* sp. was investigated in batch culture to determine their optimal N/P ratio and nitrogen and phosphorus recovery under the varying N/P ratios. Second, the kinetic coefficients of nitrogen and phosphorus uptake by the four species were determined to compare their nutrient uptake capacity and select those strains with extreme nitrogen or phosphorus removal rate.

Chapter 4 The outdoor growing benthic algal community can vary greatly in its species composition and biomass production over seasons. Thus, a 1 m^2 scale Algal Turf Scrubber (ATS) was set up by inoculating natural biofilms from a local wastewater treatment plant to follow the algal community composition, growth curve, biomass production and the biochemical composition over seasonal variations in Belgium. Furthermore, the first coming species may have long-lasting priority effects on the algal community composition and thus the biomass production, nutrient removal and biochemical composition of the biomass. In this chapter, three benthic filamentous algae *Stigeoclonium* spp. and *Pseudanabaena* sp. with high nitrogen and phosphorus removal capacity (from Chapter 3) were inoculated to separate ATS lanes to exploit their priority effects on the periphyton community formation, biomass production and nutrient removal.

Chapter 5 Different algal species have specific habitats and tolerances of wastewaters, so monoculture of *Klebsormidium* sp. and *Stigeoclonium* spp., wastewater-born algae and their mixture were cultivated in horticultural wastewater to investigate their capacity of purifying the real wastewater and their competition with the wastewater-born algae. Moreover, phosphorus showed a sharp decrease in Chapter 4 and precipitation was probably the mechanism. Thus, real horticultural wastewater and synthetic wastewater with chelating agent (EDTA) were used to compare phosphorus removal process.

Additionally, flow rate was proved to be a potential factor in benthic algal community composition and biomass production in Chapter 4. Thus, four different flow rates were set on the Algal Turf Scrubber at the same time to investigate their effects on the algal composition, biomass production and nutrient removal rate of the benthic algal community with an initial inoculum of a mixture of *Klebsormidium* sp. and *Stigeoclonium* spp.

Chapter 6 provides a general discussion of results of the previous chapters. It also gives recommendations for further research and application tips of benthic filamentous algae and their communities in the practical wastewater treatment, nutrient recovery and producing biomass with a high content of certain biochemical component.

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Chapter 2

Changes in protein content and fatty acid composition of four filamentous green algae during growth and nitrogen deprivation

Liu, J., Vanormelingen, P., Vyverman, W. Changes in fatty acid profiles of four filamentous green algae in response to growth phases and conditions, in preparation.

Abstract

Although benthic filamentous algae have a high potential for wastewater treatment and the production of algal feedstock for biotechnological applications, relatively little is known about their biochemical composition and variation therein in response to growth conditions. Protein content and fatty acid composition of four filamentous green algae with contrasting macronutrient requirements were determined at different growth phases and after nitrogen deprivation or dark exposure. *Uronema* sp. had a protein content of 49% of dry weight in exponential phase while the others had 29-37%. Culture age, nitrogen deprivation and dark exposure increased both total fatty acid content (TFA) from 12-35 to 40-173 mg g^{-1} dry weight and the relative proportion of polyunsaturated fatty acids (PUFAs) from 21-58% to 55-87% of TFA. However, the main variation in fatty acid composition was between species, with *Klebsormidium* spp. rich in C18:2ω6 (30-60% of TFA), *Stigeoclonium* sp. in C18:3ω3 (41-64% of TFA) and *Uronema* sp. in C16:0 (30-53% of TFA). A careful selection of both culture conditions and filamentous algal strains can enhance the production of proteins and certain fatty acids.

Key words: Filamentous green algae, growth phase, nitrogen deprivation, polyunsaturated fatty acid, protein

1. Introduction

In recent years, microalgae have received increasing attention in biotechnology research with regard to wastewater treatment and the production of proteins and polyunsaturated fatty acids (PUFAs) as human and animal nutrition (Babuskin et al., 2014; Cole et al., 2015). To date, most studies focused on planktonic species grown in suspension in photobioreactors or open pond systems. However, harvesting planktonic algae is estimated to account for 20-30% of the total cost of microalgal biomass cultivation. Moreover, unicellular algae are usually palatable for small zooplankton which may cause productivity losses or even the crash of mass cultures (Guo et al., 2014). In contrast, biofilm-based production systems using benthic filamentous species are easier to harvest and more resistant to small grazers due to their attached growth, large cell/colony size and thick cell wall with a high cellulose content (Guo et al., 2014; Wellnitz & Ward, 1998). However, their nutritional properties, in particular protein content and PUFAs composition, have rarely been evaluated (Praveenkumar et al., 2012; Saito et al., 2010).

Cultivation conditions (e.g. nutrient supply, light regimes, pH and salinity, etc.) can have significant influences on algal growth and biochemical composition and the effects are species-specific (Gardner et al., 2011; Griffiths et al., 2012; Hu et al., 2008; Kirrolia et al., 2011; Liu & Vyverman, 2015; Napolitano, 1994). Among nutrients, nitrogen limitation is a key factor triggering the reduction of protein content and the accumulation of lipids and fatty acids of some microalgae, while for some others it doesn't work (Bona et al., 2014; Cha et al., 2011). In the study of Griffiths et al. (2012), nitrogen limitation greatly increased the lipids content of green algae *Chlorella vulgaris* and *Scenedesmus* sp. by 2.5-4.6 times, while it had no effect on the lipids content of cyanobacterium *Spirulina platensis*. Light is a key element for photosynthesis and low light intensity favors the formation of PUFAs in many algal strains (Hu et al., 2008; McLarnon-Riches et al., 1998). Moreover, the biochemical composition is subject to variability during different growth phases (Alonso et al., 2000). Liang et al. (2006) reported that the increasing culture age caused an increase of C16:1ω7 and C18:1ω9 in *Phaeodactylum tricornutum* and C16:0 in *Chaetoceros muelleri*. Thus, an appropriate selection of algal species, manipulation of culture conditions and harvesting at certain growth stages may enable protein or PUFAs production of microalgal cultures to be tailored for specific purposes, such as producing ω-3 or ω-6 fatty acids for nutraceutical and pharmaceutical purposes (Pereira et al., 2012).

With this background, four newly isolated benthic filamentous green algae *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. and *Uronema* sp. from a wastewater treating periphyton bioreactor were cultured at different growth phases and under nitrogen deprivation or dark exposure. The changes in protein content and fatty acid profiles in response to growth phases and culture conditions were investigated and their potentials as protein or fatty acid sources in mass production or wastewater treatment were evaluated.

2. Material and methods

2.1 Algal strains and culture conditions

Four benthic filamentous green algae were isolated from a small-scale outdoor Algal Turf Scrubber (ATS). Periphyton biofilm collected from a municipal wastewater treatment plant (Aquafin, Destelbergen, Belgium) was used as an inoculum for the ATS. The steps for isolation and purification of algal strains were as described previously (Liu & Vyverman, 2015). The purified strains were enriched and kept at 23 °C in a climate room in 250 ml Erlenmeyer flasks containing 100 ml WC medium without vitamins addition or pH adjustment.

The four species were identified to genus level as *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. by their morphological features (Fig. 2.1). Subsequently, subcultures of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 for genetic analysis were harvested from exponential phase by centrifugation, and DNA was extracted following Zwart et al. (1998) using a bead-beating method with phenol extraction and ethanol precipitation. The primers NS7m and LR1850 (Friedl, 1996) were used for PCR amplification of ITS1, 5.8S and ITS2 regions of *Klebsormidium* spp. 18S rDNA of *Stigeoclonium* sp. LJ1 was amplified with primers E2 from Van Hannen et al. (1999) and P4 from Moon-van der Staay et al. (2000) after a PCR amplification of ITS regions of *Stigeoclonium* sp. LJ1 failed. The PCR amplification conditions were: 5 min at 94 °C, 35 cycles of 2 min at 60 °C, 3 min at 68 °C and 15 min at 72 °C. The resulting PCR products were analyzed on an automated ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Waltham, USA). Sequence similarity searches were performed using a nucleotide BLAST search in GenBank, and the newly generated sequences deposited in GenBank (accession numbers KR092194, KP165132 and KR002183). All strains were kept at 23 °C in a climate room in WC medium (Guillard & Lorenzen, 1972) without vitamin addition or pH adjustment, a

12:12 h light/dark cycle and a light intensity of 80-90 µmol photons m^{-2} s^{-1}. The strains *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 were submitted to BCCM/DCG culture collection (www.bccm.belspo.be, accession numbers: DCG0640, DCG0641 and DCG0642).

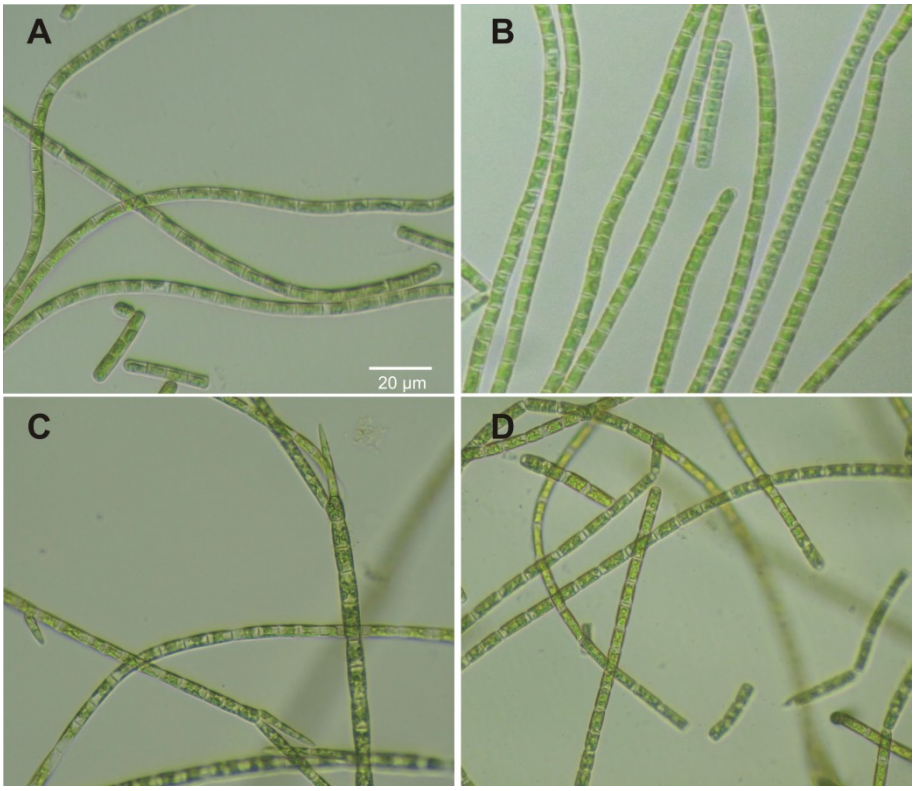

Fig. 2.1 A-D: Pictures of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. in exponential phase cultures.

2.2 Experimental design

To investigate the effects of growth phase and nitrogen deprivation on protein and fatty acid content and fatty acid composition, 20-30 ml of exponentially growing culture of each species was inoculated into a 250 ml Duran jar (with lid, Duran®) with 150 ml WC medium at an initial fluorescence value of 0.05 (F_0, fluorescence measurement in section 2.3). For each species, 9 replicate cultures were established and grown at 23 °C under a 12: 12 h light/dark cycle at a light intensity of 80-90 µmol photons m^{-2} s^{-1} and the jars were randomly replaced on the shelf every day. Growth was measured daily at the same time point using *in vivo* fluorescence. After 7 days, three replicate jars of each species were harvested, constituting the exponential phase. In three of the remaining jars the medium was replaced by nitrogen-free WC medium, while no manipulation was done on the other three jars which were used to determine stationary phase

biochemical composition. On day 12, the cultures of *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1 under nitrogen-deplete treatment reached stationary phase and were harvested. For *Uronema* sp. this was done on day 15. On day 15, the *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1 cultures under nutrient-replete condition reached stationary phase. Half of each replicate culture was harvested and the other half was covered with aluminum foil and kept in the dark for three days before harvesting to investigate the effect of dark treatment on fatty acid composition, while this was done on day 20 for *Uronema* sp.

2.3 Sampling and data analysis

To monitor their growth phase, autofluorescence of the culture was measured every 24 h with an IMAGING-PAM Chlorophyll Fluorometer (Walz, Germany). After 15 minutes dark adaptation, fluorescence of the entire culture was measured at light intensity 3 and gain 3. The dark fluorescence yield F_0 was used as a proxy of algal biomass (Honeywill et al., 2002). The biomass was harvested by filtration through pre-weighed GF/F filters which had been burnt at 550 °C for 2 h. The harvested biomass was freeze-dried overnight and weighed to obtain the dry weight (DW) and then preserved at -20 °C for further analysis.

Additionally, to evaluate the potential signal saturation of the fluorescence measurement, a calibration curve at a broad range of biomass density was constructed as below. Exponentially growing cultures of each strain were filtered through GF/F filters and then different amounts of wet cells (0.01-0.6 g) were weighed and inoculated to 150 ml WC medium in 250 ml Duran jar. Three days later, the F_0 value of each jar was measured and then the biomass was harvested by filtering through pre-weighed GF/F filters and freeze-dried to measure its dry weight. Thus, a calibration curve of F_0 and dry weight was constructed for each species (Fig. S2.1).

The specific growth rate of each species was calculated on each day from the F_0 values through Equation 2.1 and the maximal value was used as their maximal growth rate.

$$\text{Specific growth rate } (\mu, \text{ division } d^{-1}) = \frac{\log_2 F_{t2}/F_{t1}}{t_2 - t_1} \quad (2.1)$$

In Equation 1, F_{t1} was the F_0 value on day t_1; F_{t2} was the F_0 value on the next day t_2; t was the cultivation time, day.

Protein content of samples from the exponential and stationary phase and nitrogen-deplete cultures was determined. Proteins were extracted with lysis buffer from the lyophilized biomass and measured with a spectrophotometer at 562 nm following the Bicinchoninic Acid (BCA) Method (Smith et al., 1985; Stoscheck, 1990). Two technical replicates were made and the results averaged.

Fatty acid (FA) composition of samples from the exponential and stationary phase, nitrogen-deplete and dark treated cultures was determined. FAs were extracted and methylated to fatty acid methyl esters (FAMEs) following the one-step derivatization method by Abdulkadir and Tsuchiya (2008). The GC-MS analysis was carried out on an Agilent 6890/5973 GC-MS system. Subsequently, FAMEs components were identified by direct comparisons of their mass spectral pattern to the mass spectral database and their retention times to those of 37 known standard fatty acids (Supelco® 37 component FAME mix). The quantification of FAs was done by converting peak area of the chromatogram through the conversion coefficient factors obtained from the standard mixture of 37 fatty acids.

2.4 Statistical analysis

Statistical analyses were done in STATISTICA 7.0. One-way ANOVA was used to test for growth rate differences between the four species. Two-way ANOVA was used to test for differences in protein and fatty acid content between different growth phases, nitrogen conditions and the four species. Post-hoc Tukey tests were used to identify statistically significant pairwise differences. The significance level was $p = 0.05$ overall. Principal component analysis (PCA) was performed using Canoco 4.5 (Microcomputer Power, Ithaca, NY, USA) to visualize the main variation in algal fatty acid composition in response to different algal species, growth phases and cultivation conditions. Before analysis, the 7 fatty acids with insignificant amounts (< 1% of TFA) were omitted (Kumari et al., 2013) and the average relative abundances of the 10 most important fatty acids (> 1% of TFA) were square root transformed.

3. Results

3.1 Growth rate

Klebsormidium sp. LJ2 had the highest maximum specific growth rate of 0.66 ± 0.13 (average ± 1 S.D., n = 3, the same below) division d^{-1}, while *Stigeoclonium* sp. LJ1

had the lowest specific growth rate of 0.4 ± 0.03 division d^{-1}. *Klebsormidium* sp. LJ1 and *Uronema* sp. had an intermediate growth rate of 0.58 ± 0.1 and 0.46 ± 0.06 division d^{-1} respectively. The one-way ANOVA showed a significant effect of species on maximal growth rate (p = 0.02). The Post-hoc Tukey test indicated that the growth rate of *Stigeoclonium* sp. LJ1 was lower than that of both *Klebsormidium* spp.

3.2 Protein content

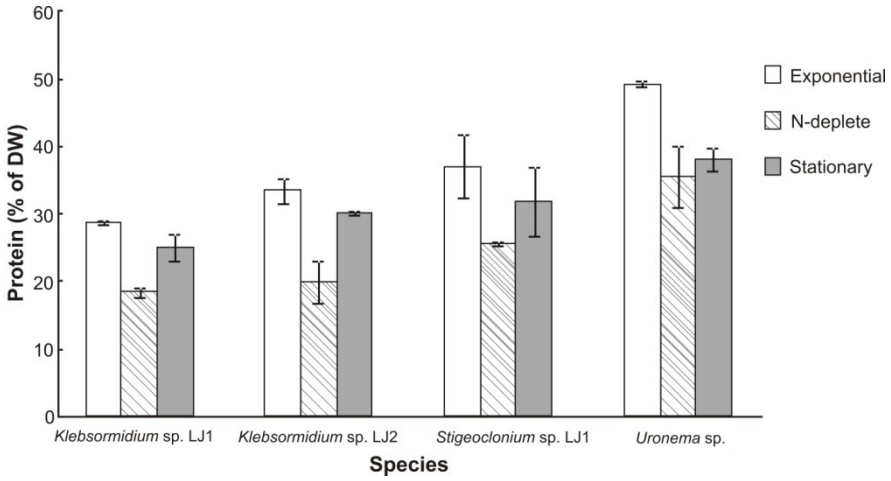

Fig. 2.2 Protein content (% of DW) of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. from exponential phase, nitrogen deprivation and stationary phase. The error bars correspond to the standard deviation of triplicates.

Overall, the protein content was the highest in exponential phase and the lowest under nitrogen deprivation. Specifically, protein contents were 29, 34, 37 and 49% of DW in exponential phase and 19, 20, 27 and 36% of DW in the nitrogen-deplete condition for *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. respectively (Fig. 2.2). The two-way ANOVA showed that the effects of species (F = 92.4, $p < 0.001$) and growth phase (F = 75, $p < 0.001$) on protein content were highly significant. The interaction effect was much smaller but also highly significant (F = 10.3, p = 0.004), indicating that the effect of growth phase on protein content depended somewhat on the species considered. The Post-hoc Tukey test showed that the species effect was mainly caused by the lower or higher protein content of *Klebsormidium* sp. LJ1 and *Uronema* sp. respectively compared to that of the other species. Concerning the growth phase, *Uronema* sp. showed significant difference in its protein content from exponential and stationary phase (p = 0.024), while the other three species didn't differ significantly (p = 0.41-1).

3.3 Fatty acid content and composition

From the GC chromatograms of *Klebsormidium* spp., *Stigeoclonium* sp. LJ1 and *Uronema* sp., 17 fatty acids were identified with C16:0, C18:2ω6 and C18:3ω3 being the major components (on average over a quarter of TFA, Fig. 2.3, Table S2.1). Generally, exponential phase samples had the lowest TFA content and those from the dark treatment had the highest. Specifically, *Stigeoclonium* sp. LJ1 and *Uronema* sp. had higher fatty acid content (32-35 mg g^{-1} DW) than *Klebsormidium* spp. (12-21 mg g^{-1} DW) during exponential phase. Dark treatment of stationary phase cultures significantly increased the fatty acid content of the four species (Fig. 2.3B), especially *Stigeoclonium* sp. LJ1, the fatty acid content of which increased from 52.1 ± 2.0 to 173.0 ± 5.9 mg g^{-1} DW. The two-way ANOVA showed that there was a highly significant species ($F = 13.3$, $p < 0.001$) and growth condition ($F = 151.1$, $p < 0.001$) effect on fatty acid content, with the latter being by far the most important factor. Furthermore, also the interaction between growth condition and species was highly significant ($F = 14.3$, $p < 0.001$). The Post-hoc Tukey test indicated that the growth condition effect was mainly caused by the low fatty acid content of the cultures from exponential phase and the high fatty acid content in the dark treatment. The species effect was mainly due to the high fatty acid content of *Stigeoclonium* sp. LJ1.

C14:0, C16:0 and C18:0 were the main saturated fatty acids (SFAs) representing 36-66% of TFA content in exponential phase (Fig. 2.3A). Although the C16:0 content of the samples from stationary phase (5.9-19.1 mg g^{-1} DW) was higher than that of exponential phase (4.6-16.8 mg g^{-1} DW), its contribution to TFA during exponential phase (24.7-53.4%) was significantly higher than during the stationary phase (18.5-31.7%). The mono-unsaturated fatty acids (MUFAs) were mainly C16:1ω7 and C18:1ω9 with a content ranging from 0 to 8 mg g^{-1} DW, contributing 0-14.8% of TFA (Fig. 3A). *Stigeoclonium* sp. LJ1 and *Uronema* sp. had a higher content of C16:1ω7 and C18:1ω9 than the *Klebsormidium* spp. C18:2ω6 and C18:3ω3 were the main PUFAs and also the major components of the TFAs (42-87%) of *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1 under all the tested conditions (Fig. 2.3A). The PUFA content of the four species in stationary phase (19-53 mg g^{-1} DW) was higher than in exponential phase (5-21 mg g^{-1} DW). *Stigeoclonium* sp. LJ1 had a much higher C18:3ω3 content than the other three species, especially the samples from dark treatment (111 ± 6 mg g^{-1} DW) (Fig. 2.3B). For *Uronema* sp., the percentage of the PUFAs in TFAs was lower than the other three species and ranged between 20% and 55% (Fig. 2.3A). Overall, increasing culture age, nitrogen deprivation and dark treatment increased both PUFAs content and their relative

contribution to the TFA content of the four species, especially for *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1.

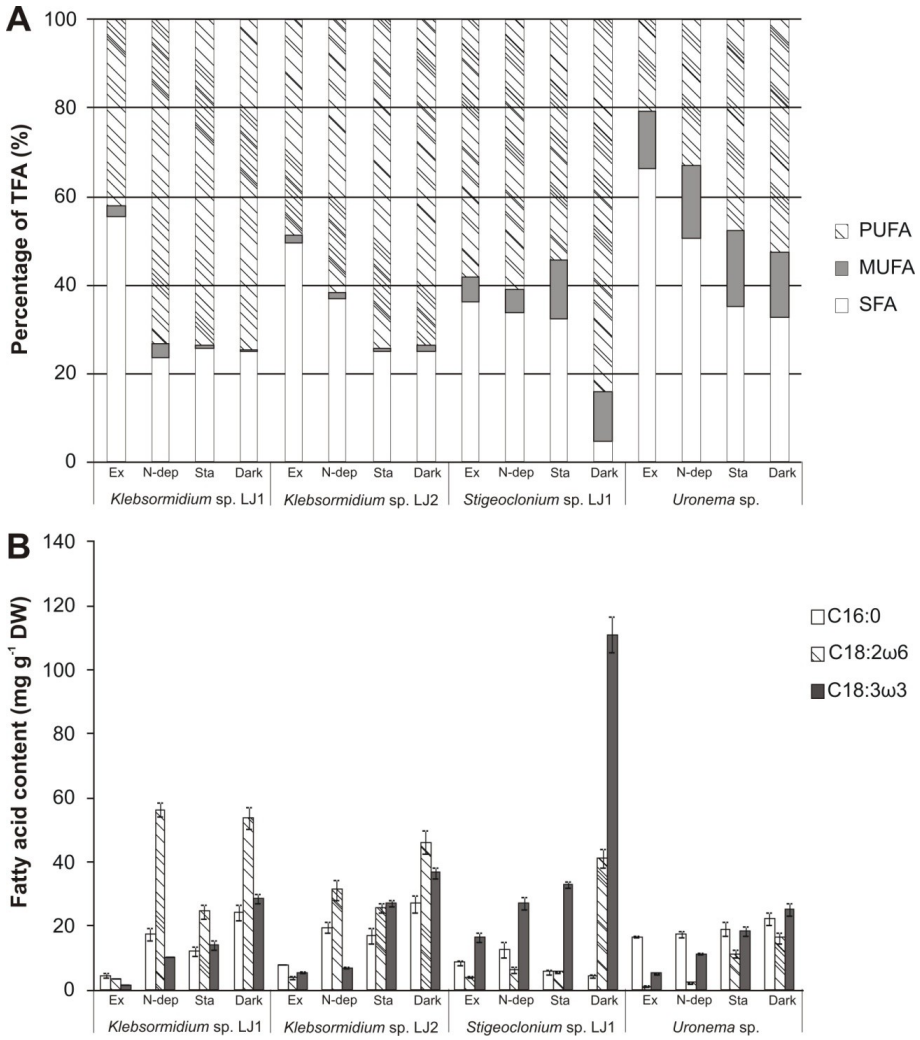

Fig. 2.3 A: Percentage composition (%) of saturated fatty acid (SA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) in total fatty acid (TFA), B: Content (mg g^{-1} DW) of C16:0, C18:2ω6 and C18:3ω3 of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. from exponential and stationary growth phases, nitrogen deprivation and dark treatment.

3.4 Multivariate Analysis of fatty acid composition

A PCA using the percentage contribution of the 10 most important fatty acids (listed in Table S2.1) was performed to visualize the main variation in fatty acid composition in relation to species identity and growth phase. PC1 accounted for 50.6% of the total variation and PC2 for 28.7%. A biplot of these two axes is shown in Fig. 2.4. The major fatty acid components underlying PC1 were C14:0, C16:1ω7, C18:1ω9, C18:2ω6, C18:3ω6 and C20:4ω6, while PC2 mainly represents variations in C16:0, C18:0 and

C18:3ω3 (Fig. 2.4), with positive scores for most PUFAs and negative scores for SFAs. The main variation in fatty acid composition was due to species differences rather than growth phase since the first two PCA axes separated the species. *Klebsormidium* spp. samples were placed in the left quadrants and *Stigeoclonium* sp. LJ1 and *Uronema* sp. in the right. Specifically, *Klebsormidium* spp. contained C18:3ω6, C20:4ω6 and C22:0, while these were not detected in *Stigeoclonium* sp. LJ1 and *Uronema* sp., and had a higher contribution of C18:2ω6 than the other species. Moreover, *Klebsormidium* spp. had a higher content of C18:2ω6 and a lower content of C14:0 than *Stigeoclonium* sp. LJ1 and *Uronema* sp.

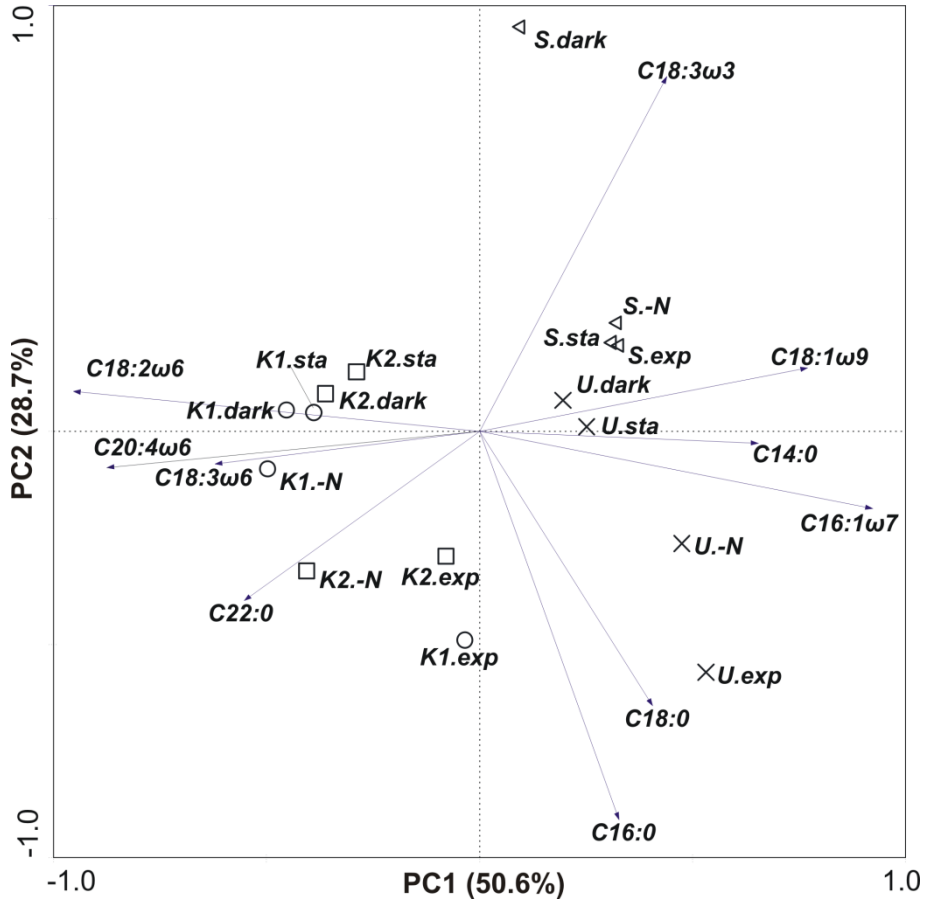

Fig. 2.4 Biplot of a principal component analysis (PCA) of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp., biplot of 10 fatty acids from exponential phase, nitrogen-deplete, stationary phase and dark treated conditions. *K1.*: *Klebsormidium* sp. LJ1; *K2.*: *Klebsormidium* sp. LJ2; *S.*: *Stigeoclonium* sp. LJ1; *U.*: *Uronema* sp.; *exp*: exponential phase; *-N*: nitrogen-deplete; *sta*: stationary phase; *dark*: dark treatment.

Klebsormidium sp. LJ1 and *Klebsormidium* sp. LJ2 had similar fatty acid profiles under the tested cultivation conditions. For these two species, the samples from the

dark treatment and stationary phase differed from the samples from exponential phase and nitrogen-deplete condition as the former samples had a lower content of C16:0 and a higher content of C18:3ω3 than the latter samples. The sample of *Stigeoclonium* sp. LJ1 in the dark treatment was distinct from other samples of *Stigeoclonium* sp. LJ1 because of its extraordinary high C18:3ω3 percentage (64%). *Uronema* sp. had a high SFA content, especially C16:0 (maximally 53.4% of TFA) and its samples were mainly separated along the second axis. The *Uronema* sp. sample from exponential phase differed from the samples of the other conditions due to its high C16:0 content.

4. Discussion

Nitrogen availability has been reported as an important factor of the growth and biomass production of microalgae, reflecting differences in nitrogen requirement of the studied algal species (Liu & Vyverman, 2015). After the medium was refreshed with nitrogen-free medium, it showed no significant decrease in the growth of the four species, especially *Klebsormidium* sp. LJ2 and *Uronema* sp. (Fig. S2.2). For *Klebsormidium* sp. LJ2, this was probably caused by the underestimation of biomass due to the saturation of fluorescence measurement. It can be seen in Fig. S2.1 that the slope of the calibration curves at the low biomass density was larger than at high biomass density. Moreover, *Uronema* sp. had a high protein content (49% of DW) during exponential phase, which equals a nitrogen content of 8-12% of DW (Cole et al., 2015). Thus, when the algal cells of *Uronema* sp. were switched to nitrogen-free condition, the high internal nitrogen content could probably support cell growth until it decreased below the subsistence quota of nitrogen (Droop, 1983; Liu & Vyverman, 2015).

It is generally accepted that fatty acid composition of microalgae depends on growth phase and nitrogen availability (Hu et al., 2008; Praveenkumar et al., 2012). In this study, a decrease in the proportion of SFAs in TFAs and a concomitant increase in PUFAs were observed from exponential phase to stationary phase and from nitrogen-replete to nitrogen-deplete conditions (Fig. 2.3A). It was in accordance with the report of Praveenkumar et al. (2012) on *Stigeoclonium* sp. that nitrogen starvation induced an increase of UFAs (unsaturated fatty acids) from 40% to 52% of TFAs. However, this was in contrast to the fatty acid composition changes of the green microalgae *Auxenochlorella protothecoides* and *Nannochloropsis* sp. and the diatom *Phaeodactylum tricornutum* where increasing culture age and nitrogen limitation were followed by an increase of the proportion of SFAs and a decline in PUFAs (Liang et al., 2006; Pasaribu et al., 2014; Recht et al., 2012).

Light intensity and light/dark cycles have significant effects on algal growth and biochemical composition and low light intensity has been shown to favor the formation of PUFAs for many algal species (Hu et al., 2008; Sharma et al., 2012). In this study, dark treatment of the stationary phase cultures of the four species increased the total fatty acid content by 30-230% (Table S2.1). Generally, the dark treatment not only increased the TFA content but also resulted in an increased proportion of PUFAs, especially C18:2ω6 and C18:3ω3 (Fig. 2.3). This was in accordance with the report of McLarnon-Riches et al. (1998) on *Selenastrum capricornutum* where dark treatment increased the proportion of C18:2ω6. Similarly, in a study of Napolitano (1994), the C18:3ω3 content of the filamentous alga *Cladophora* sp. was sensitive to changes in irradiance and its proportion of TFAs increased significantly from 12 to 26% when the light intensity changed from 1500 to 30 μmol photons m^{-2} s^{-1}.

Additionally, C18:3ω3 and C18:2ω6, which are the precursors for the biosynthesis of all other ω-3 and ω-6 PUFAs cannot be synthesized by vertebrates, are classified as essential fatty acids for human and animals (Pereira et al., 2012). In the current study, the extremely high content of C18:2ω6 in *Klebsormidium* spp. and C18:3ω3 in *Stigeoclonium* sp. LJ1 indicated their potential of being good candidates as sources of essential fatty acids. Moreover, C18:2ω6 and C18:3ω3 can be regarded as fatty acid markers of *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1 respectively (Sushchik et al., 2010).

The growth rate, metabolism and biochemical composition of different algal species can vary greatly under different cultivation conditions (Griffiths et al., 2012). In this study, *Uronema* sp. had a high protein content of 49% in the exponential phase, while the other three only had 29-37%. Furthermore, the fatty acid profiles of the four species responded differently to growth conditions. For example, dark treatment greatly increased the content of C18:3ω3 of *Stigeoclonium* sp. LJ1 from 31 to 111 mg g^{-1} DW (Fig. 2.3B), while for *Klebsormidium* sp. LJ1, LJ2 and *Uronema* sp. it slightly increased from 14 to 29, 27 to 37, 19 to 25 mg g^{-1} DW respectively. Moreover, dark treatment greatly increased the content of C16:0 of *Klebsormidium* spp. by 10-12 mg g^{-1} DW, while for *Stigeoclonium* sp. LJ1 it decreased by 1.4 mg g^{-1} DW (Fig. 2.3B). The contrasting responses to growth conditions stressed the importance of selection of appropriate species for producing biomass with high content of proteins or fatty acids (Griffiths et al., 2012).

5. Conclusions

The four benthic filamentous green algae responded differently to growth phase and nitrogen availability in their protein content and fatty acid profiles. Compared to the other species, *Uronema* sp. had the highest protein content and its growth was less influenced by nitrogen deprivation. Saturated fatty acids, especially C16:0, were the main component of these four species during exponential phase, while increasing culture age, nitrogen deprivation and dark treatment greatly increased both the content of polyunsaturated fatty acids and their proportion of total fatty acids, especially C18:2ω6 and C18:3ω3. *Klebsormidium* spp., *Stigeoclonium* sp. LJ1 and *Uronema* sp. can be good potential sources of the essential fatty acids C18:2ω6, C18:3ω3 and proteins respectively. Moreover, a better understanding of their biochemical composition can also benefit in their applications in wastewater treatment as producing biomass rich of protein or certain fatty acids.

Acknowledgements

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Supplementary information

Fig. S2.1 A-D: The calibration curve of the dark fluorescence yield (F_0) and dry weight (mg) of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. in 150 ml WC medium in 250 ml Duran jar at light intensity 3 and gain 3 with IMAGING-PAM Chlorophyll Fluorometer.

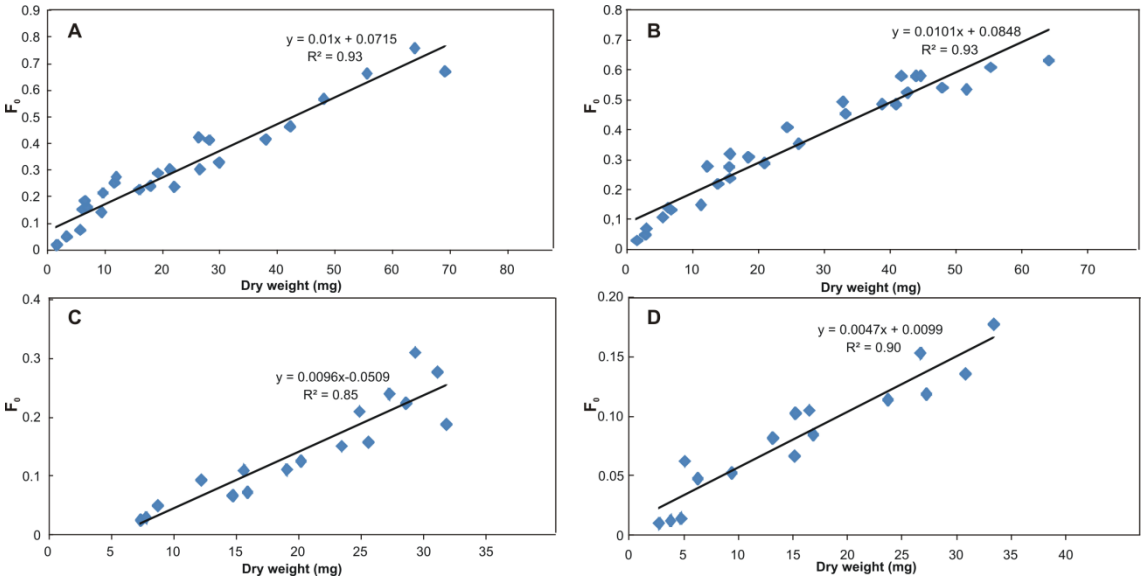

Fig. S2.2 A-D: F_0 changes of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. with and without nitrogen deprivation over time. The error bars correspond to the standard deviation of three replicates.

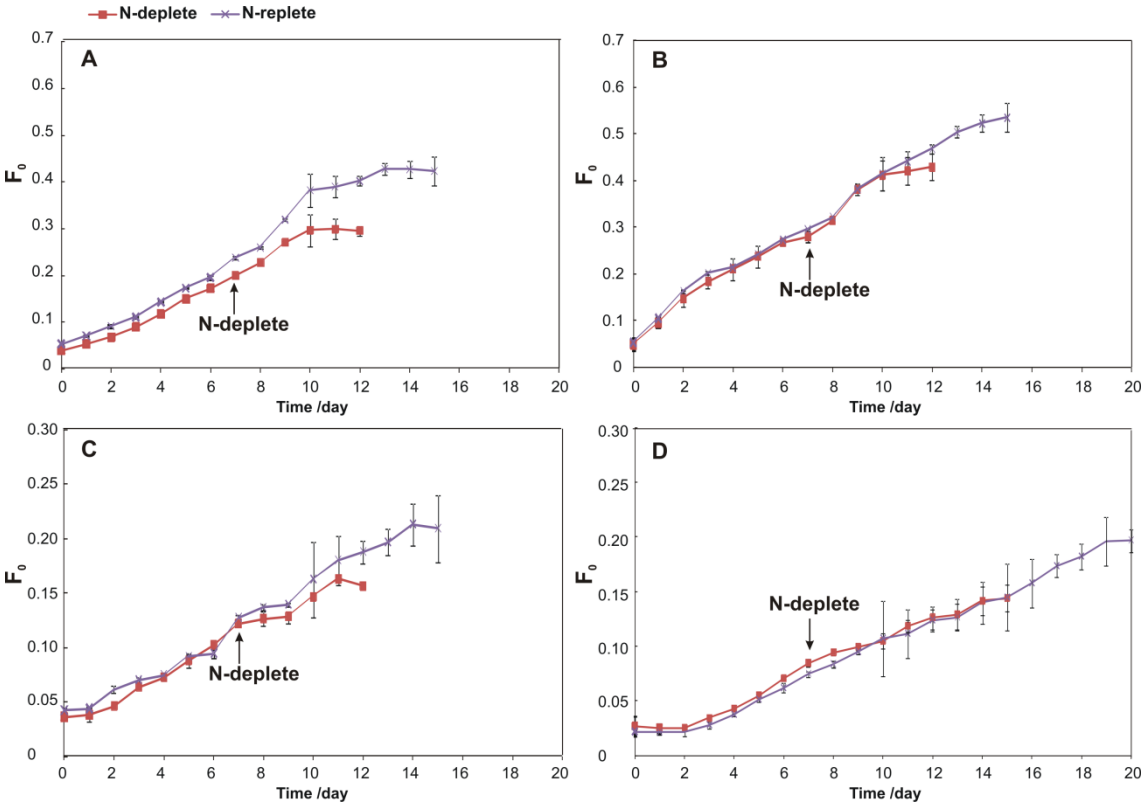

Table S2.1 Fatty acid profiles (mg g^{-1} dry weight, average ± 1 S.D., n=3) of *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. from exponential phase, nitrogen-deplete, stationary phase and dark treatment. Only fatty acids which were > 1% of TFA were included. n.d., not detected.

Species	Condition	C14:0	C16:0	C18:0	C22:0	C16:1ω7	C18:1ω9	C18:2ω6	C18:3ω3	C18:3ω6	C20:4ω6	Total
Klebsormidium sp. LJ1	Exponential	0.3 ± 0.1	4.7 ± 0.8	1.5 ± 0.3	0.2 ± 0.04	0.1 ± 0.03	0.3 ± 0.02	3.5 ± 0.1	1.5 ± 0.1	n.d.	n.d.	12.0 ± 1.8
	N-deplete	1.9 ± 0.4	17.5 ± 1.9	2.4 ± 0.2	0.4 ± 0.03	n.d.	2.8 ± 0.2	56.4 ± 2.3	10.2 ± 0.1	n.d.	2.3 ± 0.2	93.8 ± 3.3
	Stationary	1.0 ± 0.1	12.1 ± 1.5	1.3 ± 0.3	0.4 ± 0.03	0.2 ± 0.08	0.3 ± 0.1	24.7 ± 2.2	14.1 ± 1.3	0.9 ± 0.1	2.5 ± 0.2	57.2 ± 3.8
	Dark	2.0 ± 0.1	24.3 ± 2.5	2.2 ± 0.5	0.8 ± 0.05	0.2 ± 0.04	0.2 ± 0.1	53.8 ± 3.3	28.7 ± 1.3	n.d.	6.2 ± 0.4	119.0 ± 5.7
Klebsormidium sp. LJ2	Exponential	0.4 ± 0.1	7.8 ± 0.1	1.8 ± 0.3	0.3 ± 0.03	0.4 ± 0.1	n.d.	4.0 ± 0.4	5.6 ± 0.3	0.3 ± 0.03	0.8 ± 0.1	21 ± 2.9
	N-deplete	1.0 ± 0.1	19.7 ± 1.5	2.8 ± 0.1	0.5 ± 0.08	0.3 ± 0.02	0.9 ± 0.7	31.4 ± 2.9	7.0 ± 0.2	1.5 ± 0.2	0.9 ± 0.1	66.9 ± 4.3
	Stationary	0.5 ± 0.1	17.1 ± 2.3	1.7 ± 0.1	0.4 ± 0.07	0.3 ± 0.2	0.3 ± 0.1	25.7 ± 1.4	27.1 ± 0.8	3.1 ± 0.2	2.5 ± 0.2	79.4 ± 4.2
	Dark	0.6 ± 0.1	27.1 ± 2.7	2.6 ± 0.1	0.6 ± 0.09	0.6 ± 0.1	0.5 ± 0.1	46.3 ± 3.8	36.8 ± 1.7	3.8 ± 0.3	3.5 ± 0.1	123.0 ± 4.9
Stigeoclonium sp. LJ1	Exponential	2.7 ± 0.1	8.7 ± 0.8	1.3 ± 0.1	0.1 ± 0.02	0.7 ± 0.1	1.3 ± 0.01	4.1 ± 0.2	16.6 ± 1.3	n.d.	n.d.	35.4 ± 3.4
	N-deplete	3.7 ± 0.4	12.6 ± 2.5	2.0 ± 0.4	0.3 ± 0.02	0.9 ± 0.2	2.0 ± 0.6	6.4 ± 0.8	27.3 ± 1.9	n.d.	n.d.	55.3 ± 3.2
	Stationary	2.4 ± 0.3	5.9 ± 0.7	3.1 ± 0.6	n.d.	1.2 ± 0.3	2.8 ± 0.2	5.9 ± 0.3	33.1 ± 1.1	n.d.	n.d.	52.1 ± 2.0
	Dark	1.7 ± 0.2	4.5 ± 0.6	2.1 ± 0.5	n.d.	1 ± 0.2	13.6 ± 1.3	41.2 ± 3.0	111.0 ± 5.5	n.d.	n.d.	173.0 ± 5.9
Uronema sp.	Exponential	1.2 ± 0.1	16.8 ± 0.4	2.9 ± 0.1	n.d.	1.2 ± 0.1	2.8 ± 0.1	1.3 ± 0.2	5.3 ± 0.2	n.d.	n.d.	31.5 ± 1.0
	N-deplete	1.2 ± 0.2	17.6 ± 1.0	2.2 ± 0.1	0.2 ± 0.02	1.4 ± 0.1	3.4 ± 0.3	2.4 ± 0.2	11.4 ± 0.2	n.d.	n.d.	40.0 ± 2.3
	Stationary	1.2 ± 0.2	19.1 ± 2.1	1.6 ± 0.1	n.d.	1.4 ± 0.3	6.9 ± 0.9	11.5 ± 1.3	18.6 ± 1.4	n.d.	n.d.	60.4 ± 1.9
	Dark	1.5 ± 0.2	22.5 ± 1.8	1.7 ± 0.1	n.d.	1.6 ± 0.2	7.1 ± 0.3	16.5 ± 1.7	25.2 ± 1.85	n.d.	n.d.	76.3 ± 3.0

Chapter 3

Differences in nutrient uptake capacity of the benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and *Pseudanabaena* sp. under varying N/P conditions

Modified from: **Liu, J.,** Vyverman, W., 2015. Differences in nutrient uptake capacity of the benthic filamentous algae *Cladophora* sp., *Klebsormidium* sp. and *Pseudanabaena* sp. under varying N/P conditions. *Bioresource Technology*, **179**, 234-242.

Abstract

The N/P ratio of wastewater can vary greatly and directly affect algal growth and nutrient removal process. Four benthic filamentous algae species *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and *Pseudanabaena* sp. were isolated from a periphyton bioreactor and cultured under laboratory conditions at varying N/P ratios to determine their ability to remove nitrogen and phosphorus. The N/P ratio significantly influenced the algal growth and phosphorus uptake process. Optimal N/P ratios for nitrogen and phosphorus removal were 7-10, 5-12, 5-15 and 7-20 for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively. Within these respective ranges, *Stigeoclonium* sp. LJ2 had the highest biomass production, while *Pseudanabaena* sp. had the highest nitrogen and phosphorus content. This study indicated that *Stigeoclonium* sp. LJ2 had a high capacity of removing phosphorus from wastewaters of low N/P ratio, and *Pseudanabaena* sp. was highly suitable for removing nitrogen from wastewaters with high N/P ratio and high nitrogen concentration.

Keywords: N/P ratio, Nutrient uptake, *Klebsormidium*, *Stigeoclonium*, *Pseudanabaena*

1. Introduction

Human activities have caused tremendous volume of urban, agricultural and industrial wastewater production and have greatly increased the input of primary nutrients, such as nitrogen and phosphorus as well as pollutants into natural water bodies in the past decades (Abdel-Raouf et al., 2012; Aslan & Kapdan, 2006; Boelee et al., 2011; Li et al., 2010). Worldwide, eutrophication of freshwater aquatic ecosystems results in the loss of key species and ecosystem functions and the deterioration of surface water quality. As a consequence, combating eutrophication and pollution of aquatic resources is increasingly being implemented in water policy regulations. For example, the EU Water Framework Directive's objective to achieve good chemical and ecological status for all surface waters by 2015 requires improved wastewater treatment systems to further mitigate the nitrogen and phosphorus emissions (Boelee et al., 2011).

Traditional nitrogen and phosphorus removal techniques including sludge treatment, denitrification pond, and chemical precipitation of phosphorus generate a great volume of sludge waste, release nitrogen to the atmosphere and use additive chemicals (Craggs et al., 1996; Renuka et al., 2013). Others, like reed bed sewage systems and constructed wetlands, require large area of land and are low in nutrient removal capacity (Kern & Idler, 1999; Vrhovšek et al., 1996). Several studies have demonstrated the potential of algae for nitrogen and phosphorus removal (Arbib et al., 2013; Aslan & Kapdan, 2006; Boelee et al., 2011; Chinnasamy et al., 2014; de-Bashan & Bashan, 2010). Algae offer the advantages of having high growth rates and being capable of assimilating nitrogen and phosphorus from wastewater with low operational costs, less land requirement, no secondary pollution, efficient recycling of nitrogen and phosphorus, no requirement of organic carbon and no CO_2 emission (Abdel-Raouf et al., 2012; Aslan & Kapdan, 2006; Boelee et al., 2011; Renuka et al., 2013). Algae have already been used in treating secondary effluent, agricultural, domestic, piggery and dairy wastewater (Abdel-Raouf et al., 2012; Mulbry et al., 2010; Zamalloa et al., 2013; Zhu et al., 2013).

The commonly studied algae for wastewater treatment are planktonic microalgae, such as *Chlorella* and *Scenedesmus* (Aslan & Kapdan, 2006; Li et al., 2010; Zhu et al., 2013), but their harvest is time-consuming and accounts for about 20-30% of the total costs of microalgal biomass cultivation and therefore remains a major obstacle for its large-scale application (Wang et al., 2013). Recently, research efforts have increasingly

focused on using non-planktonic algae, with high surface attachment, flotation capacity, or self-aggregation capacity which can greatly reduce harvesting costs. Particularly, due to their larger size, filamentous algae such as *Cladophora*, *Oedogonium*, *Spirogyra* and *Tribonema* are attractive because they are much easier and thus cheaper to harvest (Olguin et al., 2003; Roberts et al., 2013; Wang et al., 2013). For example, in a small scale Algal Turf Scrubbers of Mulbry et al. (2010), which were dominated by the filamentous algae *Enteromorpha* sp., *Lyngbya* sp. and *Spirogyra* sp., the biomass was easily harvested by a wet/dry vacuum followed by dewatering by sieving through a 2 mm nylon netting, and resulting in a 10% solids content of concentrated biomass.

Besides the harvesting cost, another important issue for wastewater treatment is to optimize the removal efficiency of multiple nutrients (Silva-Benavides & Torzillo, 2012). Several studies using algae have attempted to increase nutrient removal by acting on the algal physiology, including algae pre-starvation before inoculation to wastewater, acclimatization to wastewater, addition of CO_2, optimization of light regime and pH (Chinnasamy et al., 2014; Roberts et al., 2013; Zamalloa et al., 2013; Zhu et al., 2013). Furthermore, the N/P ratio of wastewater can vary greatly from 1 to over 35 (in weight) (Renuka et al., 2013; Zamalloa et al., 2013; Zhu et al., 2013) and directly influence algal growth and nutrient uptake (Arbib et al., 2013; Leonardos & Geider, 2004; Perini & Bracken, 2014). For example, the work of Molina et al. (1991) showed that the specific growth rate of *Tetraselmis* sp. increased significantly from 0.04 h^{-1} to 0.056 h^{-1} following the change of N/P ratio from 0.5 to 5. A study of Li et al. (2010) documented that nitrogen removal efficiency of *Scenedesmus* sp. decreased significantly with N/P ratio higher than 15:1. Moreover, nitrogen or protein content of algal biomass is related to the nitrogen availability in the medium, so the wastewater of high N/P ratio can be a suitable source for protein production (Arbib et al., 2013). While in wastewater of low N/P ratio, which produced a nitrogen limiting condition, the lipid content of algal biomass can be enhanced (Arbib et al., 2013; Li et al., 2010). Thus, the N/P ratio of wastewater can also influence the biochemical composition of algal biomass and consequently its further utilization as animal feed or biofuel feedstock.

In addition to these process based approaches, attention must be paid to the selection of the appropriate species, but only a few studies have addressed this issue. Indeed, it is well-known that different algal groups differ greatly in their biochemical composition and thus their C: N: P stoichiometry may vary between phyla but also between related species (Burkhardt et al., 1999; Geider & La Roche, 2002; Ho et al., 2003; Quigg et al., 2003). For example, Ho et al. (2003) documented that under identical cul-

ture conditions two green algae *Dunaliella tertiolecta* and *Nannochloris atomus* had an N/P mole ratio of 38 and 25 respectively, while the N/P ratio of four diatom species *Ditylum brightwellii*, *Thalassiosira weissflogii*, *Nitzschia brevirostris* and *Thalassiosira eccentric* varied between 5.4 and 13.6. In study of Burkhardt et al. (1999), the N/P ratio of six diatom species ranged from 4.7 to 12.2 under nutrient-replete conditions with a CO_2 concentration of 16 μmol kg^{-1}. As a consequence of the variation in algal stoichiometry, the nutrient requirement and uptake rate are species-specific. In the study of Pedersen and Borum (1997), several macroalgae belonging to Chlorophyta including *Chaetomorpha*, *Cladophora*, *Codium* and *Ulva* had an ammonium and nitrate uptake rate ranging from 81 to 240 μmol NH_4^+-N g^{-1} dry weight h^{-1} and 9 to 30 μmol NO_3^--N g^{-1} dry weight h^{-1} respectively. These contrasting nutrient uptake rates stress the importance of selecting the appropriate species for efficient nutrient removal from wastewater.

So far, little attention has been paid to the effect of varying N/P ratios on growth characteristics, nutrient uptake capacities, and nitrogen and phosphorus content of monocultures of benthic filamentous algae. Knowledge of these properties may help in selecting a set of species adapted to a particular wastewater stream for efficiently removing nutrient at a lower cost of manipulation and further applying the produced algal biomass to feeding animals or producing biofuel.

Therefore, the primary aim of this study is to investigate the effect of varying NO_3^--N and PO_4^{3-}-P ratios on nutrient removal performance, biomass production, and nutrient composition of three benthic filamentous green algae *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and one cyanobacterium *Pseudanabaena* sp. isolated from a periphyton bioreactor in batch culture. Secondly, the optimal N/P ratio and the kinetic coefficients of nitrogen and phosphorus uptake are determined for the four species under various nitrogen and phosphorus concentrations.

2. Material and methods

2.1 Isolation of algal strains

The four benthic filamentous algae used in this study were isolated from a small-scale Algal Turf Scrubber (ATS) designed to investigate nutrient removal from horticulture wastewater as they were the common species. The steps for isolation and purification of algal strains were as follows: (1) a sample from a periphyton biofilm was diluted

in WC medium (Guillard & Lorenzen, 1972) in a petri dish and settled down overnight; (2) the next day, individual algal filaments were isolated and transferred to 24 well plates with WC medium; (3) after a week, the algal colonies were examined microscopically to select clonal strains; (4) non-clonal cultures were isolated again for further purification by repeating steps (2) and (3). All the steps were carried out under sterile conditions. The purified strains were enriched and kept at 23 °C in a climate room in 250 ml Erlenmeyer flasks containing 100 ml WC medium without vitamins addition or pH adjustment.

2.2 Algal identification

Four strains were identified to genus level as *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. by their morphological features. Subsequently, they were identified with DNA sequencing analysis.

Subculture of the strains *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. for genetic analysis was harvested by centrifugation during exponential phase, and DNA was extracted following Zwart et al. (1998) using a bead-beating method with phenol extraction and ethanol precipitation. After extraction, the primers NS7m and LR1850 (Friedl, 1996) were used for the PCR amplification of ITS1, 5.8S and ITS2 regions of *Klebsormidium* sp. and eukaryote general primers E2 from Van Hannen et al. (1999) and P4 from Moon-van der Staay et al. (2000) was used for the two *Stigeoclonium* strains under the following conditions: 5 min at 94 °C, 35 cycles of 2 min at 60 °C, 3 min at 68 °C and 15 min at 72 °C. Primer F27 from Höfle et al. (2005) was used for the PCR amplification of 16S rRNA of the cyanobacterium *Pseudanabaena* sp. The resulting PCR product was analyzed on an automated ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Waltham, USA). Sequence similarity searches were performed using a nucleotide BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The newly generated sequence was deposited in GenBank with accession numbers KP165132, KR002183, KR422334 and KR422335 for *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively.

2.3 Experimental set-up

The culture media for the experiments were based on WC medium, in which NO_3^--N and PO_4^{3-}-P was used as the sole nitrogen and phosphorus sources respectively,

and to which additional NO_3^--N and PO_4^{3-}-P were added to manipulate nitrogen and phosphorus concentration and ratio. The pH of the medium was adjusted to 7.0 by adding 4% HCl after autoclaving.

In the first experiment, the effect of eight different N/P ratios (from 1:1 to 20:1, details in Table 1) was tested on nutrient removal performance, algal growth and nutrient composition for the four species. The N/P ratios were set by changing the initial NO_3^--N or the PO_4^{3-}-P concentrations. Based on a NO_3^--N concentration of 14 mg L^{-1} in standard WC medium, additional K_2HPO_4 was added to obtain lower N/P ratio (in weight) of 7:1, 5:1, 2:1 and 1:1. Similarly, additional $NaNO_3$ was added to obtain higher N/P ratio of 10:1, 12:1, 15:1 and 20:1 with a PO_4^{3-}-P concentration of 1.5 mg L^{-1}.

Algal biomass for the experiments came from a culture in exponential phase (3 days after inoculation) of *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and *Pseudanabaena* sp. which were filtered through a GF/F filter and washed with distilled water three times to remove any nutrient from the original medium. Next, 0.04 g wet algal biomass was weighed and inoculated to 100 ml of the N/P adjusted medium in 250 ml Erlenmeyer flasks, resulting in an initial concentration of 0.4 g wet algal cells per liter. The Erlenmeyer flasks were covered with aluminum foil with pores for aeration. An additional subsample of the wet algal biomass was dried at 60 °C for 24 hours to measure its water content to determine their initial dry weight. Cultures were grown in triplicate at 23 °C under a 16 h: 8 h light dark cycle at a light intensity of 80-90 µmol photons m^{-2} s^{-1}. In parallel, 12, 12 and 6 supplemental Erlenmeyer flasks were set with the same algal biomass density (0.4 g wet cells per liter) for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively at each N/P ratio to monitor their growth curve by harvesting the biomass from three parallel flasks and measuring the dry weight every two days (more details in section 2.4). Erlenmeyer flasks were manually shaken and replaced randomly on the shelf twice a day. The experiment lasted until the concentration of NO_3^--N or PO_4^{3-}-P under most N/P ratios decreased below 1.0 mg L^{-1} and the detection limit of the photometer, respectively (10 days for *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp., 6 days for *Pseudanabaena* sp.).

In a second experiment, the effects of changing the absolute concentrations of both nutrients on nutrient removal rate were investigated for each species. This allows evaluating the nitrogen and phosphorus removal capacity of the four species by using Michaelis-Menten kinetics (Aslan & Kapdan, 2006). NO_3^--N was varied in 10 different

concentrations between 7 and 70 mg L^{-1} and PO_4^{3-}-P from 1 to 10 mg L^{-1} with a constant N/P ratio of 7 based on the result of the first experiment. The algal biomass preparation and experimental set-up was identical to the first experiment, and the experiment lasted for 24 hours.

2.4 Sample and data analysis

For the measurement of nutrient concentration in the medium, 2 ml of medium was collected daily and filtered through Whatman grade 6 paper filters. The filtrate was diluted to measure NO_3^--N and reactive phosphorus. NO_3^--N was measured with a spectrophotometer (Shimadzu UV-1601, Japan) at wavelength of 220 and 275 nm following the ultraviolet spectrophotometric screening method (APHA, 1998). The reactive phosphorus was measured with a Hanna Instrument Multiparameter Bench Photometer (HI 83214, Hanna) in accordance with the ascorbic acid method (APHA, 1998). The nutrient uptake ratio (N/P) on day t was calculated with Equation (3.1).

$$\text{Nutrient uptake ratio} = \frac{N_0 - N_t}{P_0 - P_t} \qquad (3.1)$$

Where N_0 was the concentration of NO_3^--N on day 0, mg L^{-1}; N_t was the concentration of NO_3^--N on day t, mg L^{-1}; P_0 was the concentration of PO_4^{3-}-P on day 0, mg L^{-1}; and P_t was the concentration of PO_4^{3-}-P on day t, mg L^{-1}.

To compare the nitrogen and phosphorus removal kinetics under different N/P ratios, the time it takes to reach the target effluent values of 2.2 mg L^{-1} NO_3^--N and 0.15 mg L^{-1} PO_4^{3-}-P as employed by the Dutch water boards as discharge guidelines for sensitive water bodies (Boelee et al., 2011), was calculated and expressed as $t_{N2.2}$ and $t_{P0.15}$ respectively. To this end, NO_3^--N and PO_4^{3-}-P consumption was modeled according to the Quiroga-Sales kinetic model for substrate consumption in batch reactors (Ouiroga et al., 1999).

The algal growth curve was determined by harvesting the biomass of three parallel flasks and measuring the dry weight (DW) every two days. Algal dry weight was determined by filtering the biomass through pre-weighed Whatman GF/F filters, freeze-drying overnight and weighing the dried filters with algal biomass the next day. The growth rate and mean algal biomass production during the whole experiment period was calculated by Equations 3.2 and 3.3.

$$\text{Specific growth rate } (\mu, \text{ division } d^{-1}) = \frac{log_2 DW_{t2}/DW_{t1}}{t_2 - t_1} \qquad (3.2)$$

$$\text{Mean biomass production } (P_0, \text{ mg DW } L^{-1} d^{-1}) = \frac{DW_t - DW_0}{t * V} \qquad (3.3)$$

In Equation 3.2, DW_{t1} and DW_{t2} represented the algal dry weight on day t_1 and t_2, mg; t_1 and t_2 were the cultivation time, d. In Equation 3.3, DW_t represented the dry weight of the algae on day t, mg; DW_0 was the dry weight on day 0, mg; t was the cultivation time, d; V was the medium volume, L.

The nitrogen content of algal biomass was determined with a C/N analyzer (FLASH 2000 NC Analyzer, Thermo Scientific) by using 10-15 mg lyophilized biomass harvested on day 10 for *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. and day 6 for *Pseudanabaena* sp. Phosphorus was measured with the Hanna Multiparameter Bench Photometer (HI 83214, Hanna) by dissolution of about 1 mg lyophilized algal biomass in 10 ml distilled water with a sonicator and digestion at 150 °C for 30 min in a COD Reactor (HI839800, Hanna). Two technical replicates for each replicate were made and the average of both measurements was used, and the result was expressed as % of DW.

Results from the second experiment were used to determine the nutrient removal coefficients with Michaelis-Menten kinetic (Equation 3.4) to evaluate and compare the nitrogen and phosphorus uptake capacity of these four species.

$$R = \frac{R_{max} S}{K_m + S} \qquad (3.4)$$

Where R was the nutrient removal rate, R_{max} was the maximal nutrient removal rate, S was the nutrient concentration, and K_m was the nutrient concentration at which nutrient uptake rate reaches half-maximum (Aslan & Kapdan, 2006; Perini & Bracken, 2014).

Equation (3.4) can be plotted in the form of Equation (3.5) to determine the nutrient uptake kinetic coefficients K_m and V_{max} (Aslan & Kapdan, 2006),

$$\frac{1}{R_{Xi}} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{S_0} \qquad (3.5)$$

Where R_{Xi} was the specific nutrient removal rate per unit algal biomass during the one day experiment, mg N (P) mg^{-1} DW d^{-1}; S_0 was the initial nutrient concentration, mg L^{-1}; V_{max} was the maximal nutrient uptake rate, mg N (P) mg^{-1} DW d^{-1}.

The data was expressed as mean ± 1 S.D. (standard deviation) and the mean was the average of triplicates. One-way ANOVA was used to test the difference between

different N/P ratio and within the four species by applying a significance level or *p*-value of 0.05 with STATISTICA 7.0.

3. Results and discussion

3.1 Effects of N/P ratio on algal growth and biomass production

As shown in their growth curve in Fig. 3.1 (A-D), each of the four species showed similar growth curves under the eight N/P ratios. *Stigeoclonium* sp. LJ2 reached significant higher ($p < 0.001$) biomass production of 52 ± 2 (S.D., same below) to 57 ± 3 mg DW L^{-1} d^{-1} than *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp., while *Pseudanabaena* sp. had the lowest of 20 ± 1 to 26 ± 1 mg DW L^{-1} d^{-1} under all tested N/P ratios (Fig. 3.1 and Table 3.1). *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. did not differ in biomass production under the eight N/P ratios ($p = 0.109$, 0.073 and 0.32, respectively), but *Pseudanabaena* sp. significantly differed in biomass production under the tested N/P ratios ($p = 0.012$). In terms of biomass accumulation, *Stigeoclonium* sp. LJ2 had the highest mean biomass production at N/P ratio of 7 and 10, while *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp. had their highest biomass production at N/P ratio of 5 to 7, 5 to 7 and 5 to 10 respectively.

The maximum specific growth rate (μ_{max}) differed significantly between the four species over the tested N/P ratios ($p < 0.001$). *Pseudanabaena* sp. had higher maximum specific growth rate (0.67-1.10 division d^{-1}) than *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp., while *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 had the similar growth rate of 0.43 to 0.60 division d^{-1} (Table 3.1). Both *Stigeoclonium* sp. LJ2 and *Klebsormidium* sp. LJ2 had their highest specific growth rate at N/P ratio of 5 to 7, while for *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp. it was observed at higher N/P ratio of 7 to 10 and 10 to 12 respectively.

From the maximum specific growth rate in Table 3.1, it can be concluded that *Pseudanabaena* sp. was the fastest growing species, as its specific growth rate reached 1.1 division d^{-1} at N/P ratio of 12, but its average biomass production was the lowest in the four species under all the tested N/P ratios (Table 3.1). That was probably due to the differences in their nutrient requirements. Following the consumption of nitrogen in the medium (Fig. 3.2 E), the specific growth rate of *Pseudanabaena* sp. dropped quickly from day 4 (0.8 to 1.1 division d^{-1}) to day 6 (0.15 to 0.3 division d^{-1}) as can be seen

Table 3.1 Algal growth parameters of *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and *Pseudanabaena* sp., P_0: mean biomass production (mg DW L^{-1} d^{-1} ± 1 S.D., n = 3); μ_{max}: maximum specific growth rate (division d^{-1}).

Species	N/P ratio	P_0 (mg L^{-1} d^{-1})	μ_{max} (d^{-1})
Klebsormidium sp. LJ2	1	30 ± 0.7	0.49
	2	30 ± 1.5	0.51
	5	33 ± 1.5	0.60
	7	34 ± 1.4	0.61
	10	30 ± 1.7	0.51
	12	32 ± 0.3	0.60
	15	32 ± 0.3	0.58
	20	33 ± 1.2	0.51
Stigeoclonium sp. LJ1	1	31 ± 0.6	0.53
	2	31 ± 2.0	0.57
	5	32 ± 1.9	0.52
	7	32 ± 1.3	0.56
	10	30 ± 1.3	0.56
	12	32 ± 1.4	0.46
	15	30 ± 0.8	0.43
	20	32 ± 2.8	0.46
Stigeoclonium sp. LJ2	1	55 ± 0.3	0.74
	2	57 ± 3.2	0.69
	5	52 ± 2.2	0.80
	7	55 ± 1.4	0.77
	10	56 ± 2.1	0.66
	12	54 ± 0.9	0.67
	15	53 ± 1.9	0.69
	20	55 ± 0.9	0.68
Pseudanabaena sp.	1	22 ± 0.7	0.67
	2	21 ± 1.4	0.94
	5	25 ± 1.0	0.89
	7	26 ± 0.9	0.85
	10	25 ± 1.3	1.09
	12	20 ± 0.8	1.10
	15	22 ± 1.8	0.86
	20	20 ± 0.7	0.85

from the slope of the growth curve in Fig. 3.1D. In contrast, the specific growth rate of *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. decreased gradually, and much biomass was accumulated even after NO_3^--N was consumed (Fig. 3.1A, B and C). The relationship between the growth rate and the limiting nutrient can be expressed as the equation (Droop, 1983): $\mu=\mu_{max}* (1-k_c/C)$, where μ is the specific growth rate (d^{-1}); μ_{max} is the

theoretical maximum growth rate (d^{-1}); k_c is the subsistence quota of nutrient; C is the nutrient content of algal cells, so photosynthesis can still continue until cell nitrogen falls below a threshold value, although at a reducing rate (Droop, 1983). Thus, *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. must have a lower nitrogen requirement than *Pseudanabaena* sp., their growth continued longer and more biomass was accumulated after NO_3^--N got depleted.

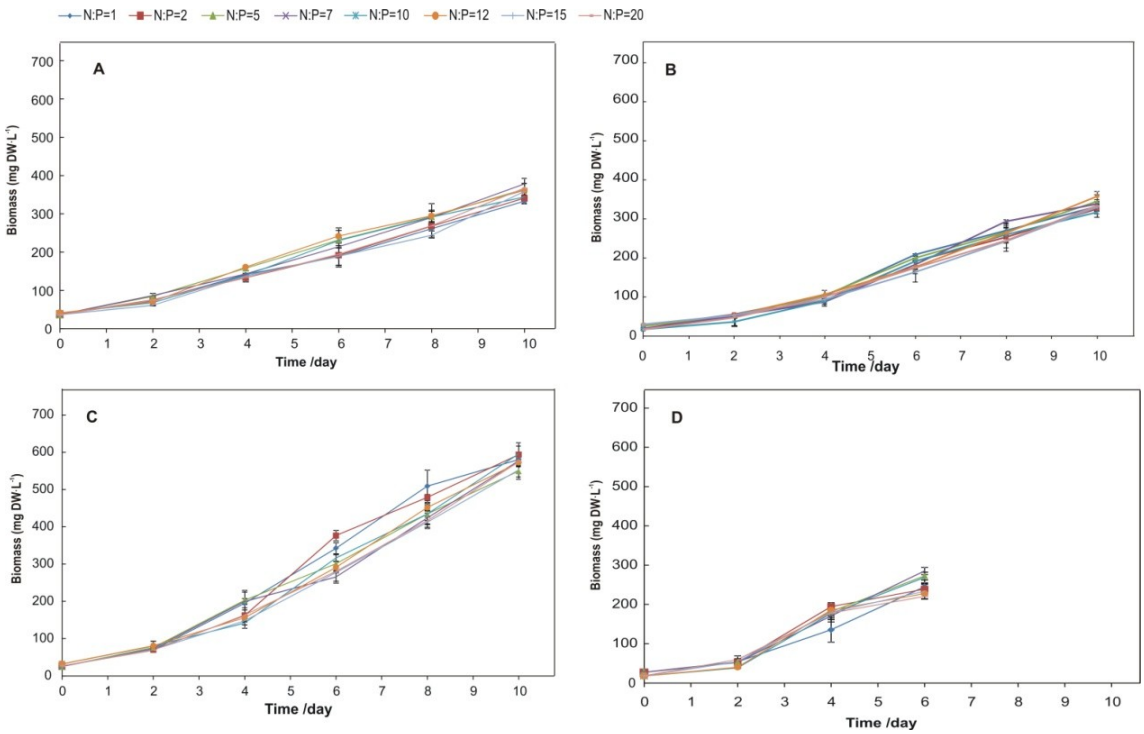

Fig. 3.1 A-D: Average biomass (mg DW L^{-1}) accumulated over time of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively under different N/P ratios of the medium. The error bars correspond to the standard deviation of triplicates.

3.2 Effects of N/P ratio on nitrate and phosphate removal

For the experiment studying the effect of different N/P ratios on nutrient removal (Fig. 3.2), *Pseudanabaena* sp. had the highest daily nitrogen removal rate (maximally 10.6 mg L^{-1} d^{-1}) and consumed the shortest time to reach the target value of 2.2 mg NO_3^--N L^{-1} (3.6 to 6.0 days, Table 3.2), while *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Stigeoclonium* sp. LJ2 removed NO_3^--N at a relatively lower rate and took longer time to reach the target value (7.0-11.2, 5.4-10.4 and 4.5-10.0 days, respectively). In terms of phosphorus removal, *Stigeoclonium* sp. LJ2 had higher phosphorus removal efficiency at low N/P ratio of 1 to 5 than the other three (Fig. 3.2B, Table 3.2), while

Pseudanabaena sp. took the shortest time to reach the target value of 0.15 mg PO_4^{3-}-P L^{-1} at high N/P ratio of 7 to 20 (3.0 to 4.0 days).

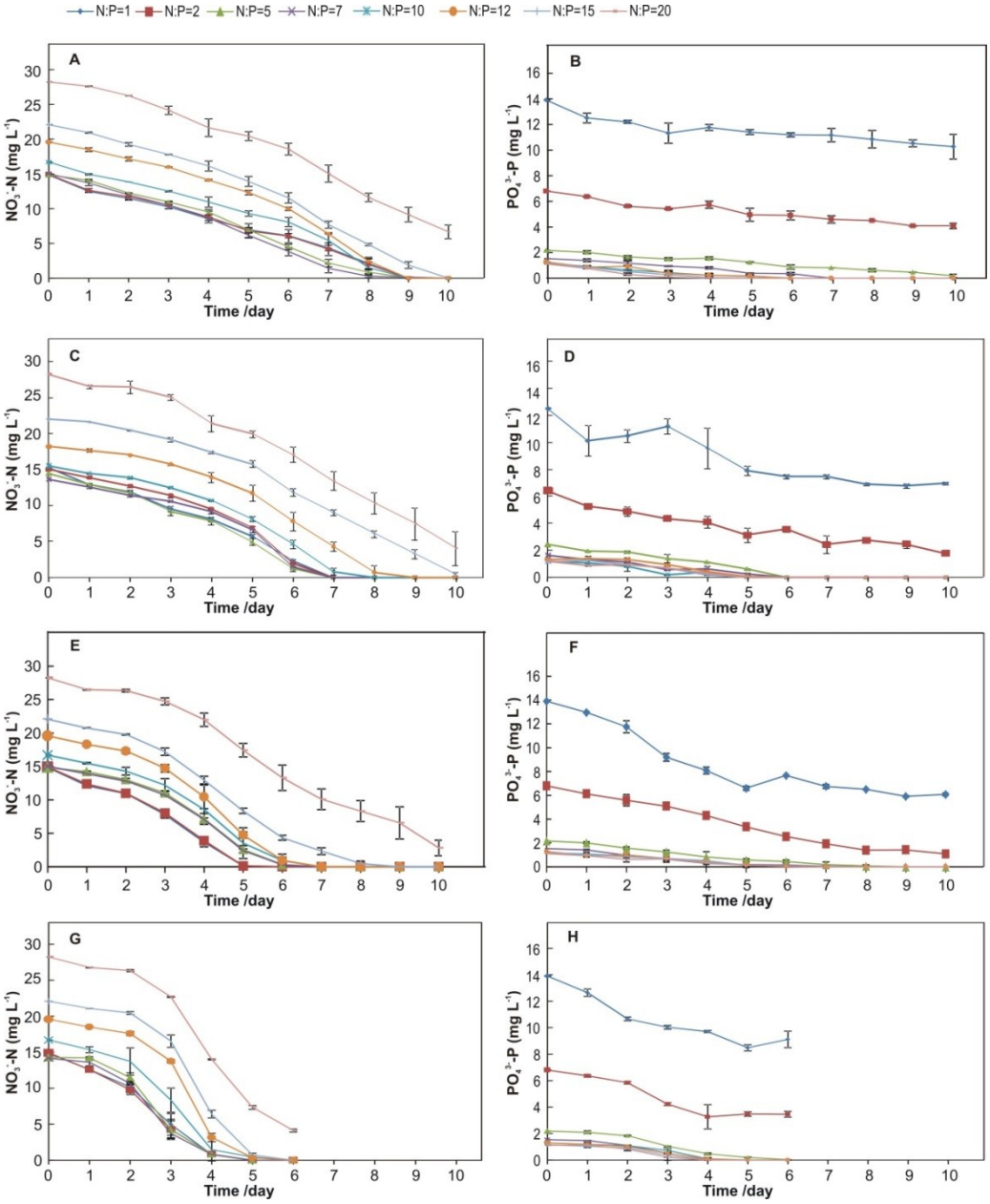

Fig. 3.2 NO_3^--N and PO_4^{3-}-P concentration (mg L^{-1}) changes in the medium with time under different N/P ratios of the four species (A, B: NO_3^--N and PO_4^{3-}-P change of *Klebsormidium* sp. LJ2; C, D: NO_3^--N and PO_4^{3-}-P change of *Stigeoclonium* sp. LJ1; E, F: NO_3^--N and PO_4^{3-}-P change of *Stigeoclonium* sp. LJ2; G, H: NO_3^--N and PO_4^{3-}-P change of *Pseudanabaena* sp. The error bars correspond to the standard deviation of triplicates.

Table 3.2 Nutrient removal efficiency and the time consumed to reach the target values of *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and *Pseudanabaena* sp. under different N/P ratios, $t_{N2.2}$, time consumed to reach 2.2 mg NO_3^--N L^{-1}, day; $t_{P0.15}$, time consumed to reach 0.15 mg PO_4^{3-}-P L^{-1}, day.

Species	N/P ratio	N removal (%)	$t_{N2.2}$ (d)	P removal (%)	$t_{P0.15}$ (d)	N/P removed ratio
Klebsormidium sp. LJ2	1	> 99	7.9	26	--	4.1
	2	> 99	7.9	40	--	5.5
	5	> 99	7.5	91	10.4	7.5
	7	> 99	6.9	> 99	6.5	9.8
	10	> 99	8.1	> 99	4.7	14.3
	12	> 99	8.3	> 99	4.8	15.5
	15	> 99	8.8	> 99	3.6	19.2
	20	76	11.2	> 99	2.8	19.4
Stigeoclonium sp. LJ1	1	> 99	6.2	45	--	2.7
	2	> 99	6.1	72	--	3.3
	5	> 99	5.8	> 99	5.8	6.0
	7	> 99	5.4	> 99	5.4	8.4
	10	> 99	6.7	> 99	4.4	13.3
	12	> 99	7.4	> 99	3.8	13.9
	15	98	9.3	> 99	4.5	18.8
	20	86	10.4	> 99	4.6	20.0
Stigeoclonium sp. LJ2	1	> 99	4.5	56	--	1.9
	2	> 99	4.5	84	11.9	2.6
	5	> 99	5.4	> 99	7.3	6.8
	7	> 99	5.4	> 99	5.5	9.8
	10	> 99	6.0	> 99	5.2	14.3
	12	> 99	6.1	> 99	5.3	15.5
	15	> 99	7.1	> 99	5.4	19.2
	20	90	10.0	> 99	5.2	22.9
Pseudanabaena sp.	1	> 99	4.0	35	--	3.1
	2	> 99	3.7	49	--	4.5
	5	> 99	3.7	98	5.3	6.9
	7	> 99	3.6	> 99	3.8	9.8
	10	> 99	4.4	> 99	3.9	14.3
	12	> 99	4.6	> 99	3.8	15.5
	15	> 99	4.8	> 99	3.6	19.2
	20	85	6.0	> 99	3.1	21.7

After 10 days of cultivation, *Stigeoclonium* sp. LJ2 removed more than 99% of NO_3^--N at N/P ratio of 1 to 15 and 90% of NO_3^--N at N/P ratio of 20. The maximal

NO_3^--N removal rate (about 4 mg N L^{-1} d^{-1}, Fig. 3.2 A) was observed during day 3 to day 5 for all the conditions, coinciding with the period of the highest specific growth rate (Fig. 3.1A). Compared to *Stigeoclonium* sp. LJ2, *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 removed NO_3^--N at a lower rate, and the maximum NO_3^--N removal rate of 2.0-2.5 and 3.0-3.9 mg L^{-1} d^{-1} was observed between day 4 and day 7 (Fig. 3.2C, E). Although more than 99% of NO_3^--N was removed at N/P ratio of 1 to 15, their nitrogen removal efficiency was 76% and 86% respectively at N/P ratio of 20. *Pseudanabaena* sp. consumed NO_3^--N in the medium much faster (maximally 10.6 mg L^{-1} d^{-1}, Fig. 3.2G) than the three green algal species and the experiment was therefore stopped on day 6. During the 6 days experiment, all NO_3^--N was removed at N/P ratio of 1 to 15 and 85% was removed at N/P ratio of 20.

Phosphorus was removed by the two strains of *Stigeoclonium* to an undetectable concentration after 10 days under N/P ratios higher than 2, while 45-72% and 56-86% of phosphorus were removed by *Stigeoclonium* sp. LJ1 and *Stigeoclonium* sp. LJ2 respectively at N/P ratios of 1 and 2 (Table 3.2). For *Klebsormidium* sp. LJ2, after 10 days of cultivation PO_4^{3-}-P was removed to an undetectable concentration at N/P ratio of 7 and higher, but the PO_4^{3-}-P removal efficiency was only 26% and 40% at N/P ratio of 1 and 2 respectively (Table 3.2). Similarly to *Klebsormidium* sp. LJ2, PO_4^{3-}-P was removed to an undetectable concentration at N/P ratio of 7 and higher by *Pseudanabaena* sp. in 6 days, and the PO_4^{3-}-P removal efficiency was 35% and 49% at N/P ratio of 1 and 2 respectively.

From the time consumed to remove nitrogen and phosphorus to the target values of 2.2 mg NO_3^--N L^{-1} and 0.15 mg PO_4^{3-}-P L^{-1} in Table 2, it can be concluded that NO_3^--N and PO_4^{3-}-P were simultaneously removed at N/P ratio of 7 within 5.5 days by *Stigeoclonium* sp. LJ2. Similarly, NO_3^--N and PO_4^{3-}-P were simultaneously reduced to the target value at N/P ratio of 7 by *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp. within 6.9, 5.4 and 3.7 days respectively. At lower N/P ratios, it took 2 more days or longer for PO_4^{3-}-P to reach the target value, and similarly the time got 1 to 4.5 days longer for NO_3^--N to reach the target value at higher N/P ratios for the four species. If the time at which NO_3^--N and PO_4^{3-}-P were removed to the target value was extended by 1 to 2 days, both of NO_3^--N and PO_4^{3-}-P can be 99% removed at N/P ratios of 5 to 15 for *Stigeoclonium* sp. LJ2, and 99% of NO_3^--N and PO_4^{3-}-P can be removed at N/P ratios of 7 to 10, 5 to 12 and 5 to 20 by *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp. respectively. Thus, the appropriate N/P ratios for the efficient NO_3^--N and PO_4^{3-}-P removal were 7 to 10, 5 to 12, 5 to 15 and 7

to 20 for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively. These N/P ratios were much higher than observed for *Scenedesmus obliquus* (4 to 5.8 in weight) (Arbib et al., 2013) and *Scenedesmus* sp. (5 to 8) (Li et al., 2010).

Fig. 3.2 illustrates that NO_3^--N uptake continued for days after PO_4^{3-}-P got depleted in the medium for the four species at high N/P ratio. In contrast, PO_4^{3-}-P uptake mainly happened before NO_3^--N got depleted in the medium for the four species, independent of how much PO_4^{3-}-P was available in the growth medium. In the case of *Stigeoclonium* sp. LJ2, NO_3^--N got depleted by day 5 at low N/P ratio and little phosphorus (less than 0.5 mg L^{-1}) was taken up afterwards (Fig. 3.2A, B). It is well known that algal nutrient uptake happens via active transport of ions across the cell membrane. For this purpose, transport proteins are necessary and they highly depend on the nitrogen availability of the growth medium (Perini & Bracken, 2014). Thus, phosphorus uptake can be suppressed by the availability of nitrogen. This is in accordance with the report of Perini and Bracken (2014) that there was a positive relationship between the tissue phosphorus content of *Fucus vesiculosus* and its ambient nitrate concentrations of the beach and the report of Beuckels et al. (2015) that nitrogen availability had a positive effect on phosphorus removal from wastewater by *Chlorella vulgaris* and *Scenedesmus obliquus*.

The final N/P removed ratio varied from 4.1 to 19.4, 2.7 to 20.0, 1.9 to 22.9 and 3.1 to 21.7 for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively following the initial N/P ratio of the medium changing from 1 to 20 (Table 3.2). This result indicates that these four species were able to adapt to a range of N/P ratios and efficiently assimilate nitrogen and phosphorus, which was in accordance with the finding of Li et al. (2010) on *Scenedesmus* sp., but the final N/P removed ratios of these four species at initial N/P ratios between 10 to 20 were higher than found for *Scenedesmus* sp. (Li et al., 2010). It also showed that *Stigeoclonium* sp. LJ2 was capable of taking up more phosphorus than the other three species. In contrast, *Pseudanabaena* sp. had a higher potential capacity of uptake NO_3^--N than the three green algae species, rendering it suitable for efficiently removing NO_3^--N from wastewaters with high N/P ratios up to 20. This feature may be due to the fact that cyanobacteria are able to store nitrogen in the form of cyanophycin granules in their cells (Renuka et al., 2013). This high nitrogen uptake capacity was in accordance with the maximal specific growth rate of *Pseudanabaena* sp. at N/P of 10 and 12 in-

stead of 5 and 7 (section 3.1) and the finding of Renuka et al. (2013) that the consortium of cyanobacteria were better able to remove NO_3^--N than the consortium of green algae.

3.3 Nutrient composition of biomass

Both nitrogen and phosphorus content of the harvested biomass of these four species were measured and shown in Fig. 3.3. In general, for the four strains, their nitrogen content slightly increased while their phosphorus content decreased significantly as a function of increasing N/P ratios. *Pseudanabaena* sp. had the highest nitrogen and phosphorus content at each tested N/P ratio.

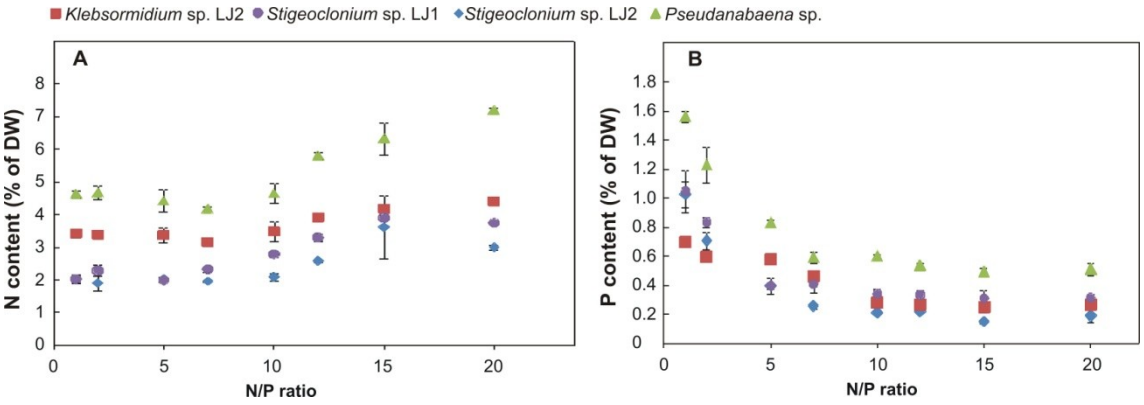

Fig. 3.3 Nitrogen and phosphorus content of the algal biomass of the four species under different N/P ratios of the medium (A: Nitrogen content, % of DW; B: Phosphorus content, % of DW). The error bars correspond to the standard deviation of triplicates.

The nitrogen content of the four species showed a similar pattern and it was relatively stable under N/P ratio of 1 to 10, varying from 3.2 to 3.5%, 2.0 to 2.3%, 1.9 to 2.1% and 4.2 to 4.7% of DW for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively. At N/P ratios above 10 the nitrogen content increased greatly up to 3.6, 3.9, 4.4 and 7.2% of DW respectively. This trend correlated well with the nitrogen uptake process and the biomass accumulation process (Fig. 3.1 and Fig. 3.2).

The phosphorous content of the four species showed similar patterns with strong decreases at N/P ratios between 1 and 7, varying from 0.7 to 0.3%, 1.0 to 0.4%, 1.0 to 0.2% and 1.5 to 0.5% of DW for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively. While the phosphorus content remained relatively stable at N/P ratios above 7, which was around 0.3%, 0.2%, 0.3% and 0.5% of DW for the four species respectively.

The decreased phosphorus content of the algal biomass and the high nitrogen removal efficiency at high N/P ratio indicated that algae can continue to grow and take up nitrogen as long as the phosphorus content of the algal cell is higher than its subsistence quota even when phosphorus gets depleted in the growth medium (Droop, 1983). This would explain why these four algae strains took up 90-100% of NO_3^--N under phosphorus limiting conditions.

3.4 Kinetics of N and P uptake

NO_3^--N uptake data of the four species *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. from the second experiment (Fig. 3.4) were plotted in form of $1/R_{Xi}$ versus $1/(NO_3^-N)_0$ and that PO_4^{3-}-P data were plotted in $1/R_{Xi}$ versus $1/(PO_4^{3-}P)_0$ (Fig. 3.5). From the slope and intercept of the best fit line of these plots (Fig. 3.5, R^2: 0.78-0.88), kinetic coefficients of NO_3^--N and PO_4^{3-}-P removal by *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. were determined (Table 3.3). *Stigeoclonium* sp. LJ2 had the highest PO_4^{3-}-P uptake rate of 27.51 mg PO_4^{3-}-P g^{-1} dry weight d^{-1}, while *Pseudanabaena* sp. had the highest NO_3^--N uptake rate of 70.6 mg NO_3^--N g^{-1} dry weight d^{-1}.

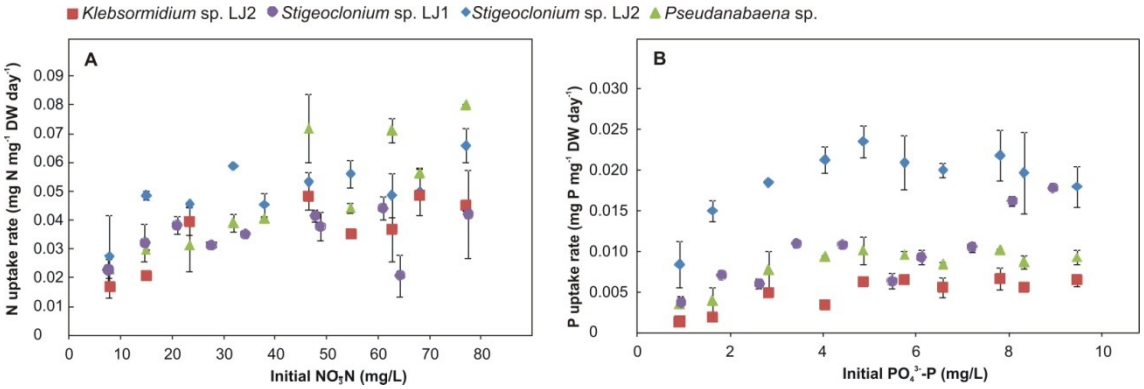

Fig. 3.4 NO_3^--N and PO_4^{3-}-P uptake rate of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. under various NO_3^--N and PO_4^{3-}-P concentrations (A: NO_3^--N uptake rate, mg N mg^{-1} DW d^{-1}; B: PO_4^{3-}-P uptake rate, mg P mg^{-1} DW d^{-1})

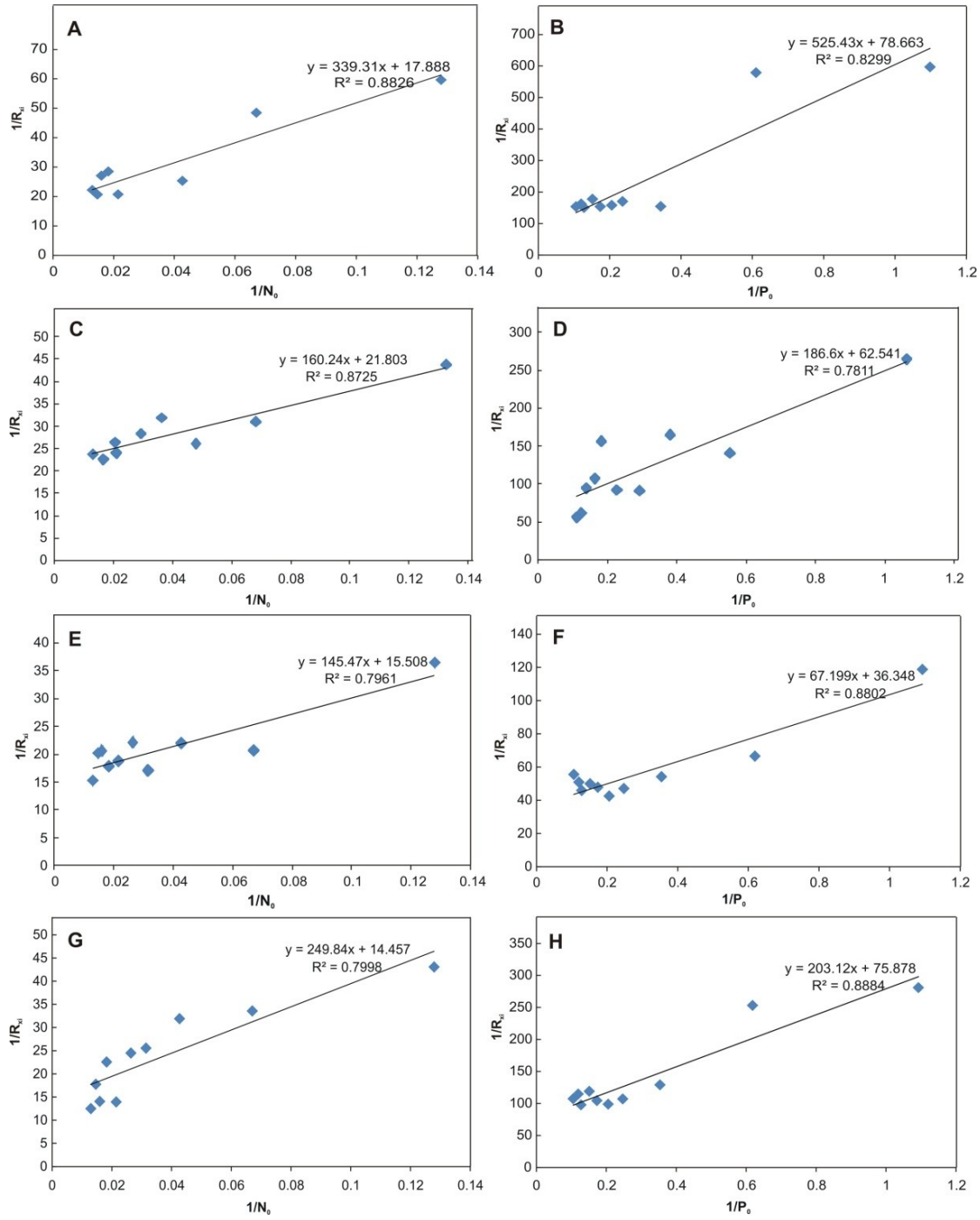

Fig. 3.5 Determination of kinetic coefficients V_{max} and K_m for NO_3^--N and PO_4^{3-}-P uptake of the four species (A, B: NO_3^--N and PO_4^{3-}-P of *Klebsormidium* sp. LJ2; C, D: NO_3^--N and PO_4^{3-}-P of *Stigeoclonium* sp. LJ1; E, F: NO_3^--N and PO_4^{3-}-P of *Stigeoclonium* sp. LJ2; G, H: NO_3^--N and PO_4^{3-}-P of *Pseudanabaena* sp.; R_{Xi}: specific nutrient removal rate, mg NO_3^--N or PO_4^{3-}-P mg^{-1} DW d^{-1}; N_0: initial NO_3^--N concentration, mg L^{-1}; P_0: initial PO_4^{3-}-P concentration, mg L^{-1})

Table 3.3 The kinetic coefficients K_m (mg NO_3^--N or PO_4^{3-}-P L^{-1}), V_{max} (mg NO_3^--N or PO_4^{3-}-P g^{-1} DW d^{-1}) and the ratio V_{max}/K_m of *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and *Pseudanabaena* sp.

Species	NO_3^--N			PO_4^{3-}-P		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Klebsormidium sp. LJ2	19.0	55.9	2.9	6.7	12.7	1.9
Stigeoclonium sp. LJ1	7.3	45.9	6.3	3.0	16.0	5.4
Stigeoclonium sp. LJ2	9.4	64.5	6.9	1.9	27.5	14.9
Pseudanabaena sp.	18.2	70.6	3.9	2.7	13.2	4.9

A high V_{max} of the algae is an indicator of the algal potential capacity of taking up nutrient, while a low K_m means that the algae species can reach its highest nutrient uptake rate at low nutrient concentrations (Pedersen & Borum, 1997; Perini & Bracken, 2014). So the ratio V_{max}/K_m can be used to compare the competitive ability of nutrient uptake of different algal species, in other words, the algal affinity for nutrient. *Stigeoclonium* sp. LJ2 had the highest V_{max}/K_m values of both NO_3^--N and PO_4^{3-}-P uptake and the highest V_{max} of PO_4^{3-}-P (Table 3.3), which indicated a competitive advantage at low NO_3^--N concentration compared to *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Pseudanabaena* sp., and generally greater capacity of taking up PO_4^{3-}-P at any concentration. The highest V_{max} and K_m of NO_3^--N uptake by *Pseudanabaena* sp. indicated its capacity of taking up NO_3^--N at high NO_3^--N concentration of the growth medium.

3.5 Potential for large-scale application

As the N/P ratio of wastewater can vary greatly (Zamalloa et al., 2013; Zhu et al., 2013), the species *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. used in this study could be good candidates for treating wastewater of low and high N/P ratio, respectively. Furthermore, these benthic filamentous algae have the advantage of growing attached to substrates. Cultivation systems designed for filamentous algae like the Algal Turf Scrubber and Rotating Algal Biofilm Reactor have been available for some time (Craggs et al., 1996; Kesaano & Sims, 2014; Mulbry et al., 2010). Extrapolating from the results of this study, pilot experiments in bioreactors designed to promote the growth of (sets of) particular species with growth and stoichiometric characteristics optimally adapted to given nutrient inputs, is the logical next step. Considering the different ecologies of benthic filamentous algae, additional issues to be addressed in upscaling include taking into account the effects of climate conditions (light and tempera-

ture regimes), substratum characteristics, influent hydraulic loading rate, grazing and disease susceptibility of the algae (de-Bashan & Bashan, 2010; Kesaano & Sims, 2014; Mulbry et al., 2010; Sandefur et al., 2014) as well as consideration of harvesting and further applications of the algae biomass, e.g. animal feed, fertilizer or biofuel feedstock.

4. Conclusions

Algal phosphorus uptake strongly depended on N/P ratio of medium, while nitrogen uptake depended less on N/P ratio. The appropriate N/P ratios for efficient nutrient removal by *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. were 7 to 10, 5 to 12, 5 to 15 and 7 to 20 respectively. *Stigeoclonium* sp. LJ2 was efficient of assimilating phosphorus from wastewater of low N/P ratio, and *Pseudanabaena* sp. was efficient in removing nitrogen from wastewater of high N/P ratio. A better understanding of the physiological diversity in growth rate, nutrient requirement and N/P stoichiometry of benthic algae and their habitat requirements can contribute significantly to the optimization of nutrient removal from wastewater.

Acknowledgements

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Chapter 4

Exploiting priority effects of benthic filamentous algae on Algal Turf Scrubber (ATS) in nutrient removal from horticultural wastewater

Liu, J., Danneels, B., Vanormelingen, P., Vyverman, W., Exploiting priority effects of benthic filamentous algae on Algal Turf Scrubber in nutrient removal from horticultural wastewater, in preparation.

Abstract

Benthic filamentous algae have extreme advantages of attaching to the substrate and ease in harvest in a wastewater treatment system. In this study, 1 m^2 scale Algal Turf Scrubbers (ATS) were set up and inoculated with natural and assembled benthic algal communities to investigate algal community composition, biomass production and nutrient removal over the seasons in a temperate climate. The ATS had a biomass production of 0.1-1.9, 0.7-4.9 and 0.2-1.6 g dry weight m^{-2} d^{-1} in spring, summer and autumn respectively. Operation of the ATS in winter was not possible due to the extremely low biomass production and ice formation. At a low flow rate, benthic algae had a longer-lasting priority effect on the periphyton community than at high flow rates, but the dominance of benthic filamentous algae showed no significant improvement in biomass production and nutrient removal. Nitrogen and phosphorus content of the produced algal biomass were 6-9% and 1.3-2.3% of dry weight respectively. This study indicated that temperature and solar irradiance had great influences on biomass production under the natural conditions in Belgium and a low flow rate could facilitate the dominance of benthic filamentous algae in periphyton.

Keywords: Algal Turf Scrubber, benthic algae, periphyton, priority effect, horticultural wastewater

1. Introduction

The great human population increase and the rapid industrialization from the middle of the 20th century have caused the continuous production of a tremendous volume of wastewaters (Abdel-Raouf et al., 2012). Although organic carbon can be removed from agricultural and domestic wastewater efficiently in wastewater treatment plants making use of bacterial communities, the remaining high concentrations of inorganic nitrogen and phosphorus in the resulting effluent can lead to eutrophication of freshwater ecosystems (Boelee et al., 2011; Shangguan et al., 2015). Therefore, many countries have set an upper limit at the inorganic nitrogen and phosphorus concentrations of wastewater that flows out into the environment, such as 10 mg TN L^{-1} and 1.0 mg TP L^{-1} of urban wastewater effluent in EU countries (Blöch, 2005), which creates a need for their removal before releasing wastewater into natural water ecosystems. One solution is to utilize the nutrient removal capacity of algae (Abdel-Raouf et al., 2012).

Algae have the ability to efficiently assimilate dissolved nutrients and convert them into proteins and other organic compounds (Cole et al., 2015; Griffiths et al., 2012; Klausmeier et al., 2004; Liu & Vyverman, 2015). Additionally, benthic filamentous algae have merits of reducing harvesting cost via attached growth to the substrate and resistance to the predation of invertebrate grazers (Biggs & Thomsen, 1995; Guo et al., 2014; Mulbry et al., 2008). Algal Turf Scrubber (ATS) is a benthic algae based cultivation system developed by Adey and coworkers in the 1980s for wastewater treatment (Adey et al., 1993). Generally, the ATS is a controlled system in which water is pumped over an inclined surface covered with periphyton (mainly composed of benthic algae, bacteria and protozoa (Larned, 2010; Wu et al., 2014)), with a periodic harvest of the photosynthetically produced biomass (Adey et al., 2011; Craggs et al., 1996; Mulbry et al., 2008). So far, ATS has wide applications in treating dairy manure, agricultural and domestic wastewater, and has been proved to be a simple-constructed and cost-efficient system compared to the wetland and photobioreactor systems (Adey et al., 2011; Chisti, 2007; Pizarro et al., 2002). Moreover, the biomass produced from ATS can be utilized as biofuel feedstock, animal feed and slow release biofertilizers (Adey et al., 2011; Mulbry et al., 2010; Mulbry et al., 2006).

The essential elements of an ATS are a solid support for periphyton growth over which a water flow is created, a source of nutrient-rich water and light (Adey et al., 2011; Craggs et al., 1996). Accordingly, the algal community composition, light intensity, temperature, hydraulic characteristics and nutrient loading are the main factors influ-

encing algal growth and nutrient removal in an ATS (Adey et al., 2011; Craggs et al., 1996; Mulbry et al., 2008; Sandefur et al., 2011; Wellnitz & Leroy Poff, 2006). As a light-driven process, the biomass production of ATS increases with increasing light levels and may reach 35-60 g dry weight m^{-2} day^{-1} (Craggs et al., 1996; Guzzon et al., 2008; Mulbry et al., 2010; Sandefur et al., 2011), but will decrease again at very high light levels due to light-induced damage in Photosystem II (photoinhibition). Temperature is another factor influencing the rate of algal photosynthesis as described by the Arrhenius Law, where algal growth increases with raising temperature up to the optimum growth temperature, and then rapidly decreases until their lethal temperature is reached (Boelee et al., 2014). For an outdoor ATS, both light and temperature show great seasonal and shorter-term weather fluctuations at higher latitudes, so seasonal variation plays a vital role in biomass accumulation and thus nutrient removal. Craggs et al. (1996) showed that in summer in central California, USA, the dry biomass production was 50-60 g m^{-2} day^{-1}, while it decreased to 8-12 g m^{-2} day^{-1} in winter. Mulbry et al. (2010) reported that nitrogen and phosphorus removal rates decreased from 0.25 to 0.016 and 0.045 to 0.003 g m^{-2} day^{-1} respectively from summer to winter. Besides the biomass production, algal community composition also showed great seasonal variations. In the study of Craggs et al. (1996), *Oscillatoria* sp., *Navicula* sp. and *Nitzschia* sp. were the main species in winter and spring, while *Cladophora*, *Microspora*, *Rhizoclonium*, *Spirogyra*, *Stigeoclonium*, *Tribonema* and *Ulothrix* became a major component of the periphyton community in summer and autumn. However, there is no report yet on an ATS in Western Europe.

As the growth rate and nutrient uptake capacity of algae vary greatly between different species, the algal community composition of an ATS can have critical effects on its nutrient removal performance (Craggs et al., 1996; Liu & Vyverman, 2015; Pedersen & Borum, 1997). Moreover, the biochemical composition of the resultant biomass from ATS can affect its further processing, such as animal or aquaculture feed, biofuel feedstock and fertilizer (Chinnasamy et al., 2014; Mulbry et al., 2005). Therefore, it would be beneficial to manipulate the algal species composition of the periphyton community on ATS in improving nutrient removal and producing valuable biomass with a high content of certain biochemical components.

In a community, the early-arriving species may have a lasting effect of colonization on the species composition of the community, which is referred to as a priority effects (Louette & De Meester, 2007; van Gremberghe et al., 2009). Specifically, the early-arriving species may gain precedence to the limiting resources including nutrients, light and space, and have a rapid population growth. Thus the carrying capacity can be

reached before the later-arriving species become abundant (Kardol et al., 2013). Second, early-arriving species can alter the environment in a favorable or detrimental way for later-arriving species (van Gremberghe et al., 2009). Thus, inoculating certain algal species to the ATS may benefit in manipulating the periphyton community composition and improving biomass production as well as nutrient removal efficiency.

Accordingly, the objectives of this study were to: (1) set up an outdoor ATS to remove nutrient (inorganic nitrogen and phosphorus) from horticultural wastewater by natural or assembled benthic algal community; (2) find out the seasonal variations in the benthic algal community composition and biomass production; (3) exploit whether priority effects can be applied to control the benthic algal community composition, and improve biomass production and nutrient removal; and (4) investigate the biochemical composition of the produced biomass, such as nitrogen, phosphorus and protein content.

2. Materials and methods

2.1 Algal Turf Scrubber set-up

The Algal Turf Scrubber (ATS) unit used in this study consisted of a water pump, a 120 L tank and a rectangular lane covered with plastic liner (see Fig. 4.1). It was located outdoors on the lawn of Campus Sterre at Ghent University, Ghent, Belgium. The size of the lane unit was 0.39 m in width and 2.5 m in length (area 0.975 m^2), and it was set at a slope of 1%. Four units including three lanes each were set up, giving a total of 12 replicate lanes.

Algal community composition, biomass production and nutrient removal and their seasonal variations were followed during three consecutive years. In July 2012, three individual lanes were inoculated with a biofilm collected from a municipal wastewater treatment plant (Aquafin, Destelbergen, Belgium), which was mainly composed of filamentous green algae, pennate diatoms, unicellular algae and cyanobacteria. Horticultural wastewater (Proefcentrum voor Sierteelt vzw, Destelbergen, Belgium) was used and the ATS lanes were inoculated with equal amount of biofilm. In October 2012, another three lanes were inoculated with a mixture of *Klebsormidium* and *Stigeoclonium* from culture to figure out their effect on the periphyton community and biomass production. Similarly, in April 2013, six lanes were inoculated with a mixture of biofilms collected from the same municipal wastewater treatment plant in 2012 and some eutrophic ponds around Ghent, and another three lanes were inoculated with

Stigeoclonium from culture. The flow rate of all those lanes was 4 L min^{-1} (water velocity 6 cm s^{-1}). In March 2014, three lanes were inoculated with natural biofilms from the same wastewater treatment plant as the last two years and the flow rate was set at 8 L min^{-1} (12 cm s^{-1}).

Fig. 4.1 View of the Algal Turf Scrubbers (ATS) used in this study

In order to determine the strength of priority effects, their effect on algal community composition, production and nutrient removal (in comparison to the natural inoculum biofilms), in June 2014, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. from culture were inoculated individually to ATS lanes in triplicate on three ATS units, and the flow rate was 8 L min^{-1}. After the first trial failed, in August 2014 the nine lanes inoculated with cultures were re-inoculated individually with the same strains *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 or *Pseudanabaena* sp. in triplicates with a lower flow rate of 2 L min^{-1} (3 cm s^{-1}). For all the three years, initially 65 L horticultural wastewater was used and continuously recirculated, and the wastewater was refreshed every three to four weeks.

2.2 Biomass production

After the inoculation, it took around two weeks for the biofilm to fully establish on the plastic foil, after which water current was produced by pumping the wastewater over the bioreactor. After running for a week, the biomass was harvested and discarded. From then on, the biomass was harvested weekly to monitor the algal community com-

position and biomass productivity. In 2012, the harvest happened from the middle of August to the end of December. In 2013 and 2014, the harvest was carried out from May to November and April to November, respectively. Because of near-zero productivity, preventing weekly harvests, and ice formation, which quickly blocked the system at freezing temperatures, the ATS was closed during winter, except the early winter of 2012.

For the harvest, the pump was switched off for a few minutes to drain the water. Then, the biomass was slightly scraped from the plastic foil by leaving a layer of about 1 mm and the biomass was collected to 500 ml plastic bottles. After that, the pump was switched on again as soon as possible to avoid drying out of the biofilm. Then the harvested biomass was centrifuged at 2000 *g* for 5 min and the wet biomass was weighed after pouring out the upper layer water. Next, a subsample was transferred to an aluminum cup, weighed to determine the fraction of the total wet weight taken, dried at 60 °C for 24 h and weighed again to measure the moisture content. This way the total biomass production in dry weight (DW, Equation 4.1) was determined. Next, the sample was burnt at 550 °C for 2 h to measure the ash content (Equation 4.2). At the same time, subsamples were collected and freeze-dried for biochemical analysis.

$$\text{Biomass production (g DW m}^{-2}\text{ d}^{-1}) = \frac{(W_5 - W_3)(W_2 - W_1)}{(W_4 - W_3) * A * N} \qquad (4.1)$$

$$\text{Ash content (\% of DW)} = \frac{W_6 - W_3}{W_5 - W_3} * 100\% \qquad (4.2)$$

In Equation 4.1 and 4.2, W_1 was the weight of the bottle, g; W_2 was the weight of bottle and wet biomass, g; W_3 was the weight of aluminum cup, g; W_4 was the weight of aluminum cup and wet biomass, g; W_5 was the weight of aluminum cup and dry biomass, g; W_6 was the weight of aluminum cup and biomass after burnt, g; A was the area of the bioreactor, m²; N was the sampling interval, day.

2.3 Growth curve determination

From May to December of 2013, three individual lanes were used to monitor the growth curve in spring (May to June), summer (July to August), autumn (September to October) and early winter (November to December). Biomass samples were collected every two or three days by scraping off the biofilm in an area of 3.5×39 cm^2 at the upper and lower portions on each lane, and then the biomass was filtered through pre-weighed

GF/F filter and oven-dried at 60 °C for 24 h. The dry weight was measured and used as proxy of biomass for the growth curve.

2.4 Community composition

On the harvesting day, subsamples of the periphyton were collected to 15 ml falcon tube and fixed with equal volume of 4% formalin for microscopical identification. The algal species were identified to genus level under light microscope and recorded semi-quantitatively in 2012 and 2013. In 2014, to assess the algal composition of the biofilms, 50 µl of the sample was transferred to a microscope slide and 9 photos were randomly taken at 400× magnifications (Leitz Diaplan Microscope, Germany), and this was done in triplicates. Then the cell numbers of each genus on each photo were counted with Image J and converted to their biomass by multiplying their biovolume, which was calculated with the equations proposed by Hillebrand et al. (1999) by measuring the sizes of 30-40 cells with Image J. The result of each genus was expressed as the relative abundance in the total biomass.

2.5 Wastewater analysis

Due to small but continuous leakage of the bioreactor in 2012 and 2013, nutrient analysis was not carried out. In 2014, the concentrations of NO_3^--N and PO_4^{3-}-P were measured every two to three days. Every time, distilled water was added or excess water from rain was removed to the 65 L level and then 15 ml water was collected. The samples were filtered through Whatman grade 6 paper filters and the filtrate was used to measure pH, NO_3^--N and PO_4^{3-}-P. pH was measured with a pH meter (PHM210, Unisense). NO_3^--N was measured with a spectrophotometer (Shimadzu UV-1601, Japan) at 220 and 275 nm following the ultraviolet spectrophotometric screening method (APHA, 1998). The PO_4^{3-}-P was measured following the Vanadomolybdophosphoric acid colorimetric method with a spectrophotometer at 400 nm (APHA, 1998).

2.6 Algal biomass content

The lyophilized biomass was crushed and preserved at -20 °C for further analysis. Carbon and nitrogen content of the biomass was analyzed with a C/N analyzer (FLASH 2000 NC Analyzer, Thermo Scientific) by using 10-15 mg lyophilized biomass. Phosphorus was measured with the Hanna Multiparameter Bench Photometer (HI 83214, Hanna) by dissolution of about 1 mg lyophilized algal biomass in 10 ml distilled

water with a sonicator and digestion at 150 °C for 30 min in a COD Reactor (HI839800, Hanna). Protein was extracted with lysis buffer overnight and measured with spectrophotometer at 562 nm following the Bicinchoninic Acid (BCA) method (Smith et al., 1985; Stoscheck, 1990). Two technical replicates for each sample were made and the average of both measurements was used, and the results were expressed as % of dry weight.

2.7 Statistical analysis

Two-way ANOVAs were used to test for statistical differences in biomass production or nutrient content of the biomass with inoculum and date as independent fixed factors (using STATISTICA 7.0). Post-hoc Tukey tests were used to determine significant pairwise differences. A significance level of $p < 0.05$ was applied throughout.

3. Results

3.1 Algal community composition

The biodiversity of the algal community in 2012 and 2013 was not high with 15 genera of green algae in total over these 8 months, and only 5 to 9 genera were found during the same season. The main genera were *Acutodesmus*, *Chlamydomonas*, *Desmodesmus*, *Klebsormidium*, *Mougeotia*, *Scenedesmus*, *Stigeoclonium* and *Melosira*, and some cyanobacteria and pennate diatoms were found as well. From Table 4.1, it can be seen that in the first few weeks of the experiment either in 2012 or 2013, the ATS lanes inoculated with *Klebsormidium* or *Stigeoclonium* were dominated by benthic filamentous algae. However, a month later, those filamentous algae were replaced gradually by the unicellular *Acutodesmus*, *Chlamydomonas*, *Desmodesmus* and *Scenedesmus*. For example, *Stigeoclonium* was successfully inoculated to the ATS lanes in May 2013, but it lost its dominance gradually from July. From October on, the temperature started to decrease from over 20 °C to less than 10 °C and several species disappeared, but *Desmodesmus* and *Scenedesmus* still remained a high quantity. Cyanobacteria had a low abundance in the beginning of the experiment, but replaced the filamentous green algae and became dominant in the summer of 2013, especially on those lanes inoculated with filamentous green algae from culture (Table 4.1).

In 2014, the algal species composition under high and low flow rate was presented in Fig. 4.2 and 4.3 respectively. As shown in Fig. 4.2, *Stigeoclonium* dominated

Table 4.1 Algal community composition on the ATS inoculated with natural biofilm and algal culture in 2012 and 2013

Species	2012										2013													
	August		September		October		November		December		May		June		July		August		September		October		November	
	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
Acutodesmus	++	n.d.	++	n.d.	++	-	++	+	++	++	+	+	++	++	++	++	++	++	++	++	++	++	++	++
Cladophora	-	n.d.	-	n.d.	-	-	+	-	-	-	+	-	+	-	++	+	++	++	++	-	++	++	-	-
Chlamydomonas	++	n.d.	++	n.d.	++	-	++	++	-	++	+	++	++	++	++	+	++	-	+	++	+	+	++	+
Cloniophora	-	n.d.	-	n.d.	-	-	+	++	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coccal green colony	+++	n.d.	++	n.d.	++	+	++	+	-	-	-	+	-	+	++	++	-	-	-	+	-	-	+	+
Desmodesmus	+++	n.d.	+++	n.d.	+++	-	+++	+	+++	-	-	-	+	-	+	-	+	-	-	-	+	-	-	+
Hormidium	+	n.d.	-	n.d.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Klebsormidium	++	n.d.	-	n.d.		++	+	++	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microspora	-	n.d.	-	n.d.	+	-	-	-	-	-	-	-	-	-	-	+	+++	+++	-	-	-	-	-	-
Mougeotia	++	n.d.	-	n.d.		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scenedesmus	++	n.d.	++	n.d.	+++	-	+++	+	+++	+++	+	+	++	+++	++	+++	++	++	+++	+++	+++	+++	+++	+++
Stigeoclonium	-	n.d.	++	n.d.	++	+++	++	+	+	-	+++	+++	+++	++	++	++	++	+	+	+	-	-	-	+
Ulothrix	-	n.d.	-	n.d.		-	-	+	-	+	+	-	+	+	++	+++	++	++	++	++	++	++	-	-
Cyanobacteria	++	n.d.	++	n.d.	++	+	++	+++	+	+++	+	++	+++	+++	+++	+++	++	++	++	++	++	++	++	++
Melosira	+++	n.d.	-	n.d.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pennate diatoms	+++	n.d.	+	n.d.	+	++	++	++	+	+	++	++	++	++	+	-	-	-	-	-	-	-	-	+

+++: dominating; ++: many; +: present; -: absent; n.d.: not determined. N: natural biofilm; C: culture.

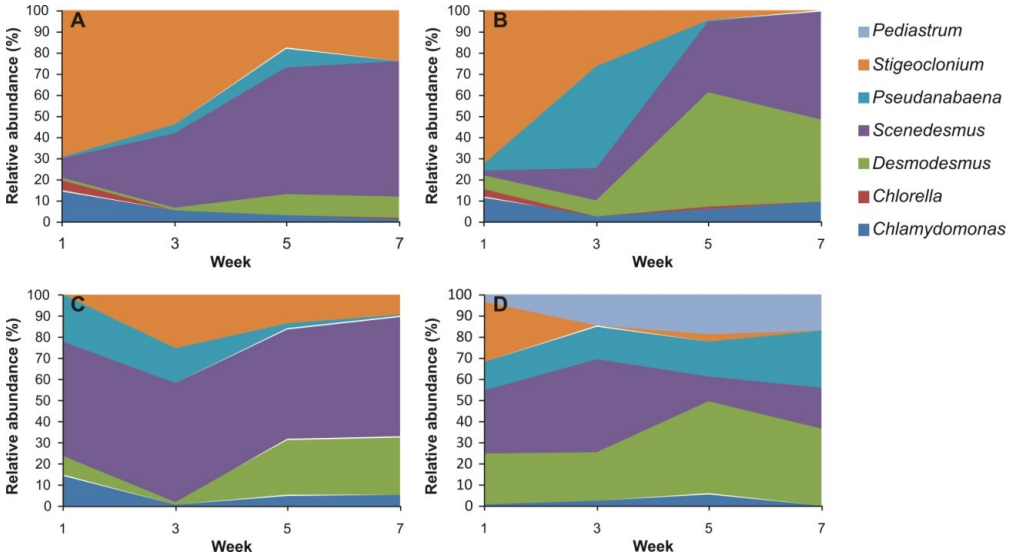

Fig. 4.2 Periphyton community composition on the outdoor ATS inoculated with *Stigeoclonium* spp., *Pseudanabaena* sp. or natural biofilm over the operating period at high flow rate in 2014 (A: *Stigeoclonium* sp. LJ2; B: *Stigeoclonium* sp. LJ1; C: *Pseudanabaena* sp.; D: Natural biofilm).

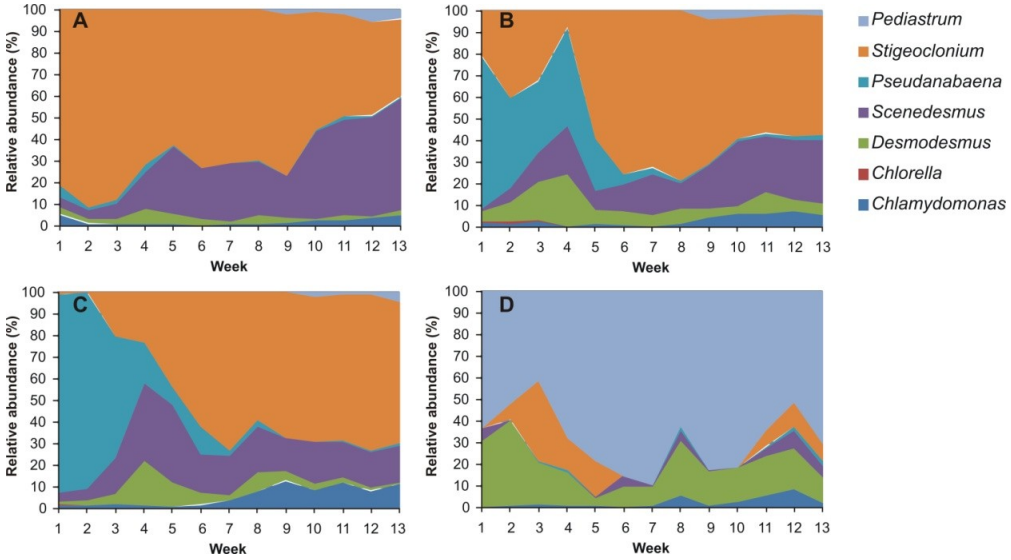

Fig. 4.3 Algal community composition of the outdoor ATS inoculated with *Stigeoclonium* spp., *Pseudanabaena* sp. and natural biofilm over the operating period at low flow rate in 2014 (A: *Stigeoclonium* sp. LJ2; B: *Stigeoclonium* sp. LJ1; C: *Pseudanabaena* sp.; D: Natural biofilm).

on the bioreactor for about two weeks with a contribution of 69-72% to total biomass. From the third week on, the relative abundance of *Stigeoclonium* decreased sharply to 27-54%. While for the lanes inoculated with *Pseudanabaena*, it lost its dominance after running one week with a relative abundance of 23%. Then its relative abundance de-

creased further to less than 5% by week 5. Accordingly, the relative abundances of *Desmodesmus* and *Scenedesmus* increased significantly from 1-9% and 3-54% in week 1 to 9-39% and 51-64% in week 7 respectively. In summary, the benthic filamentous algae were gradually replaced by *Desmodesmus* and *Scenedesmus* under a flow rate of 8 L min^{-1}.

At a relative low flow rate of 2 L min^{-1}, the benthic filamentous algae dominated on the ATS much longer than at 8 L min^{-1}, especially the lanes inoculated with *Stigeoclonium* sp. LJ2 (Fig. 4.3A). *Stigeoclonium* maintained their dominance with a relative abundance of 70-90% from September to the end of October in 2014. Then the relative abundance of *Stigeoclonium* decreased gradually to 50% by the end of November. During the same period, *Scenedesmus* increased significantly with a relative abundance from 15 to 45%. For the lanes inoculated with *Stigeoclonium* sp. LJ1, in the beginning there was contamination from *Pseudanabaena* with a relative abundance of 60%. After 5 weeks running, *Pseudanabaena* disappeared gradually and the relative abundance of *Stigeoclonium* increased from 59 to 79% (Fig. 4.3B). From the end of October (week 8), the relative abundance of *Stigeoclonium* decreased gradually while that of *Scenedesmus* increased from 12 to 30%. For these lanes inoculated with *Pseudanabaena*, they were dominated by *Pseudanabaena* in the first two weeks of the experiment (Fig. 4.3C). From the third week on, the relative abundances of *Scenedesmus* and *Stigeoclonium* increased gradually. By week 6, *Stigeoclonium* had a relative abundance of higher than 50% and maintained their dominance until the end of November. For those lanes inoculated with natural biofilms from wastewater treatment plant, *Pediastrum* was the dominating species over the whole period with a relative abundance of 42-90% (Fig. 4.3D). Therefore, the benthic algae *Stigeoclonium* had a long-lasting priority effect on the formation and structure of periphyton community, while *Pseudanabaena* only had a short period priority effect.

3.2 Growth curve of benthic algal community

After the previous harvest, the left-over periphyton forming the inoculum for the next period of growth was around 2-4 g DW m^{-2}. From the growth curves in Fig. 4.4, it showed that in the first few days the biofilms grew exponentially and the initial biomass density had significant effect on the biomass production. In the spring (May) of 2013, the maximal biomass production of the bioreactor was 3 g DW m^{-2} d^{-1} (between days 4 and 6) and the maximal algal biomass density reached 34 g DW m^{-2}. While in the summer, the biomass production reached 5 g DW m^{-2} d^{-1} (between days 3 and 9) and the

biomass density was as high as 52 g DW m^{-2}, higher than in any other season. In the autumn, the biomass production decreased significantly from the summer and it was maximally 4 g DW m^{-2} d^{-1} (between days 3 and 6), while the maximal biomass density was 27 g DW m^{-2}. In the beginning of winter, the maximal biomass production was 0.4 g DW m^{-2}. It can be concluded that maximal biomass accumulation rate was the highest in summer, followed by spring and autumn, while in early winter only a very low periphyton biomass accumulated. Concerning the biomass accumulation rate, this was significantly lower in autumn and winter than in spring and summer, so a longer harvest interval or larger inoculums would be preferred.

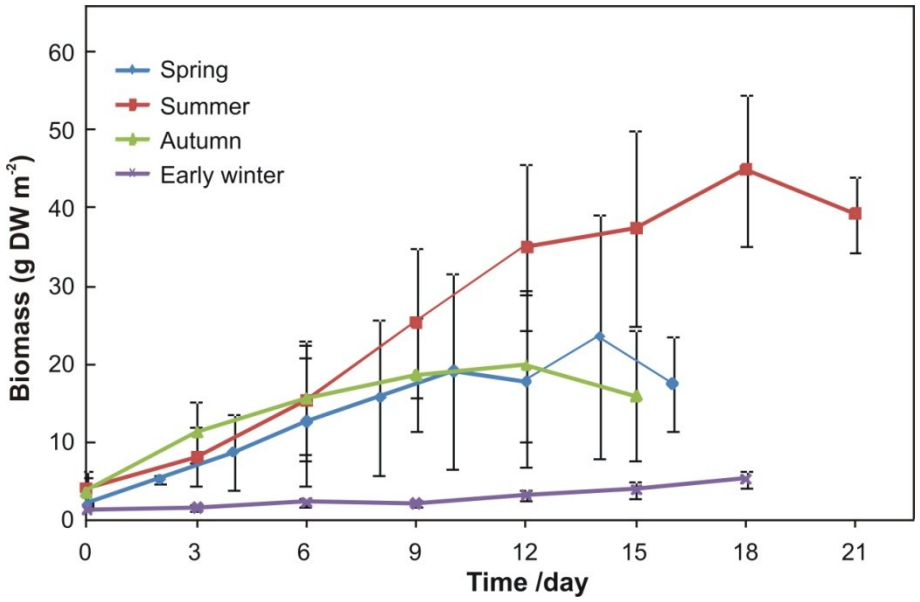

Fig. 4.4 The growth curve of the ATS inoculated with natural benthic algal community in different seasons of 2013 (spring: May to June; summer: July to August; autumn: September to October; early winter: November to December). The error bars correspond to the standard deviation of triplicates.

3.3 Biomass production

Initially in spring, biomass production was around 0.1-0.5 g DW m^{-2} d^{-1}. Thereafter, the biomass production started to increase and reached a relatively stable status (Fig. 4.5). The maximal biomass production was observed during June and September, which was 2.4 g DW m^{-2} d^{-1} and 4.9 g DW m^{-2} d^{-1} for the year 2013 and 2014 respectively. In the three years, the biomass production of the ATS started to decrease from the beginning of October and it was lower than 1.0 g DW m^{-2} d^{-1}. From the beginning of November, the biomass production decreased further to below 0.5 g DW m^{-2} d^{-1}. The two-way ANOVA showed that in 2013 there was significant difference in biomass pro-

duction between the two kinds of inoculums (F = 10.5, p = 0.002) and between the growth periods as well (F = 23.4, $p < 0.001$), but the interaction between growth period and inoculums had no significant effect on the biomass production (F = 1.5, p = 0.091). The Post-hoc Tukey test indicated that the effect of inoculums on biomass production was mainly caused by the higher biomass production of the lanes inoculated with natural biofilm than those inoculated with *Stigeoclonium* sp. The effect of growth period on biomass production was mainly caused by the higher biomass production in July to October than that in May, June and November. Similarly, the one-way ANOVA showed that there was a significant difference between the growth periods in 2014 as well (F = 5.9, p = 0.003).

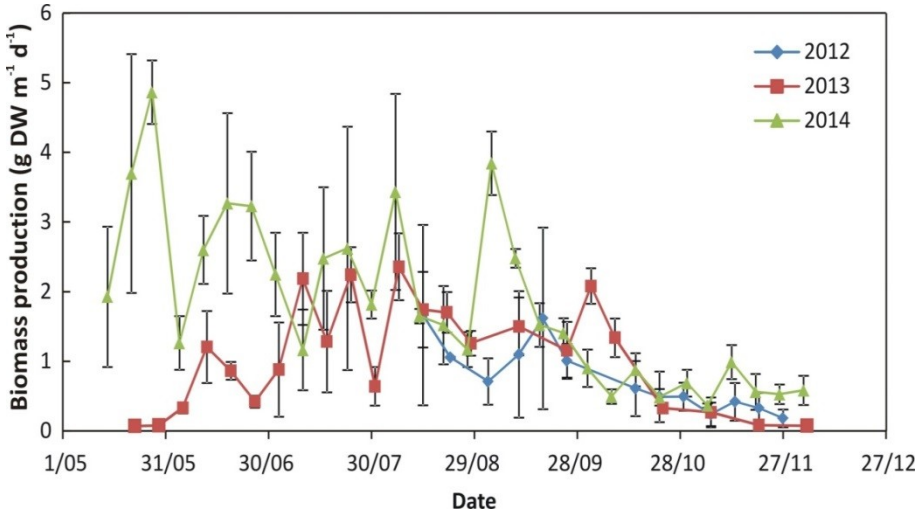

Fig. 4.5 The biomass production of the ATS inoculated with natural biofilm in 2012, 2013 and 2014. The error bars correspond to the standard deviation of triplicates.

As shown in Fig. 6, during July and August 2014 the biomass production of the lanes inoculated with *Stigeoclonium* and *Pseudanabaena* had a lower biomass production (0.7-2.6 g DW m^{-2} d^{-1}) than the lanes inoculated with natural biofilm (1.6-3.4 g DW m^{-2} d^{-1}). The one-way ANOVA showed that there was significant difference in biomass production between the inoculums (F = 4.9, p = 0.002) during this period. From September to November, all the lanes showed a similar pattern in their biomass production which varied between 0.4 and 1.4 g DW m^{-2} d^{-1}. Furthermore, the one-way ANOVA showed that there was no significant difference between the four inoculums (F = 2.2, p = 0.094).

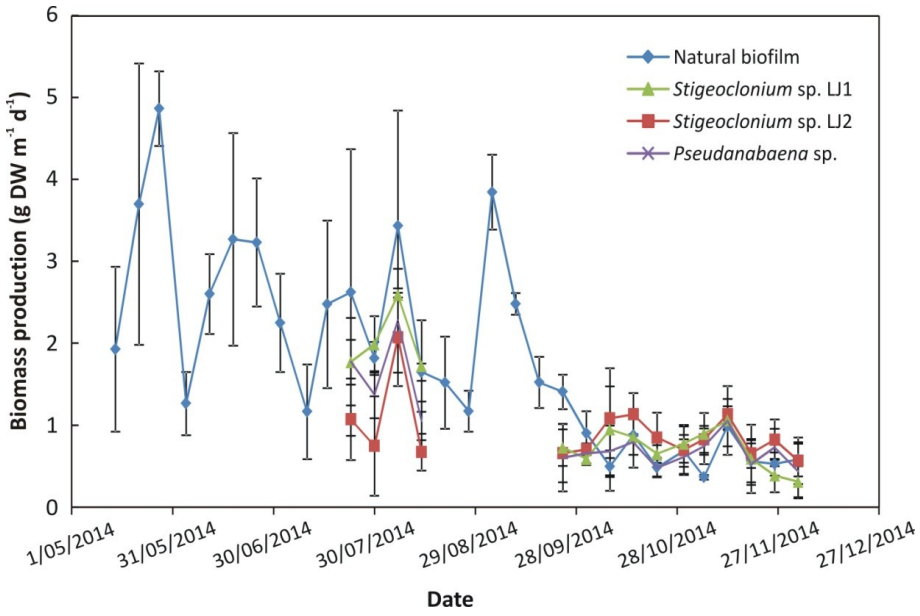

Fig. 4.6 The biomass production of the ATS with different inoculums in 2014 (*Stigeoclonium* spp., *Pseudanabaena* sp. and natural biofilm), the error bars correspond to the standard deviation of triplicates.

The ash content ranged between 13 ± 3% and 27 ± 7% of DW in the three years (Table 4.2) and this was higher than that (10% of DW) reported by Kebede-Westhead et al. (2003), but lower than that (60% of DW) of Mulbry et al. (2010). The highest ash content (26-27% of DW) mainly appeared in the beginning few weeks of the experiment. After a stable biofilm was developed on the bioreactor, the ash content was relatively stable at 13-20% of DW. The carbon content varied from 35 ± 6% to 42 ± 2% of DW in the three years. The low carbon content was observed in the beginning few weeks of the experiment, and then the carbon content stayed around 40% of DW. This was in accordance with the changes of ash content.

Table 4.2 Ash content (% of dry weight) of produced biomass in the ATS in 2012, 2013 and 2014 (n.d., not determined)

Ash content (%)	2012	2013	2014
May	n.d.	n.d.	26.9 ± 7.6
June	n.d.	26.2 ± 14.2	27.0 ± 6.8
July	n.d.	15.7 ± 5.6	22.3 ± 7.4
August	26.1 ± 1.6	20.1 ± 7.4	19.2 ± 6.1
September	27.0 ± 14.6	25.4 ± 8.2	18.6 ± 7.0
October	17.7 ± 5.9	25.1 ± 6.2	18.6 ± 8.3
November	14.0 ± 7.3	15.4 ± 3.7	13.3 ± 3.0
December	n.d.	n.d.	n.d.

3.4 Nutrient removal and recovery

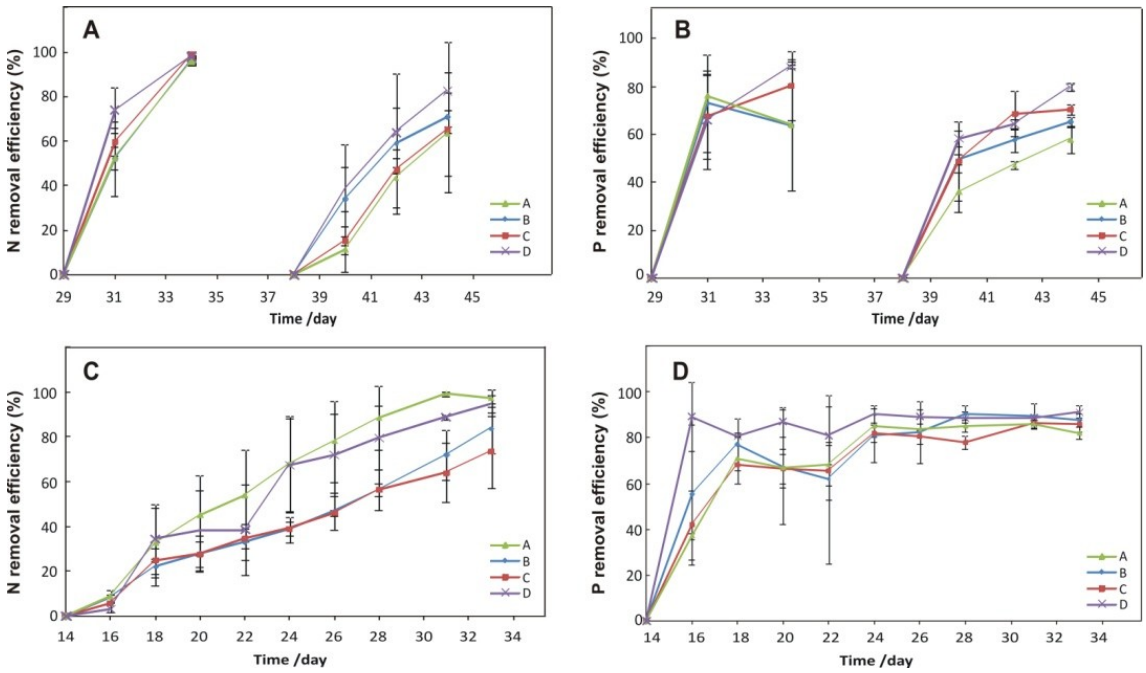

Fig. 4.7 A-D: Nitrogen and phosphorus removal efficiency of the ATS lanes with different inoculums over time in summer and autumn of 2014 (A: N removal efficiency in summer; B: P removal efficiency in summer; C: N removal in autumn; D: P removal in autumn). The error bars correspond to the standard deviation of triplicates.

In 2014, the nitrate and phosphate concentrations changes were monitored and nitrogen and phosphorus removal efficiency in the summer and autumn was shown in Fig. 4.7 (A-D). The nitrogen removal rate on the ATS showed a similar pattern with the biomass production and varied greatly in different months from 5.3 mg L^{-1} d^{-1} in July and 0.7 mg L^{-1} d^{-1} in November and the lanes with different algal communities showed a similar pattern (Table 4.3, Fig. 4.7). It took one to two weeks to reach the nitrogen discharge norm (10 mg TN L^{-1}) in Belgium. The low nitrogen removal rate was in accordance with the low biomass production of this ATS. In terms of the phosphorus removal, there was a sharp decrease in PO_4^{3-}-P concentration in the first two days after refreshing the wastewater in the tank and consequently a maximal phosphorus removal rate of 4.5-5.0 mg PO_4^{3-}-P L^{-1} d^{-1} was observed.

The nitrogen, phosphorus and protein content of the biomass produced from the ATS were sensitive to nitrogen and phosphorus concentrations of the wastewater and it varied between 5.8% and 6.3%, 1.2% and 2.6%, 22% and 42% of DW for nitrogen, phosphorus and protein respectively (Table 4.3). However, both of nitrogen and phos-

phorus recovery rates of the ATS were low with nitrogen recovery rate ranging from 50% to 63% and phosphorus recovery rate of 37-60%.

Table 4.3 Maximal NO_3^--N and PO_4^{3-}-P removal rate ($R_{max, N}$, $R_{max, P}$, mg N/P L^{-1} d^{-1}), nitrogen, phosphorus and protein content of harvested biomass (% of dry weight) and their recovery rate (%) of the outdoor ATS

Parameters	
Volume (L)	65
$R_{max, N}$ (mg N L^{-1} d^{-1})	5.3
N content (%)	5.8-6.3
N recovery rate (%)	50-63
$R_{max, P}$ (mg P L^{-1} d^{-1})	5.0
P content (%)	1.2-2.6
P recovery rate (%)	37-60
Protein (%)	22-42

3.5 Feasibility of ATS in horticultural wastewater treatment

Based on the results of this study, an average daily nutrient removal rate of 200 mg NO_3^--N m^{-2} d^{-1} and 85 mg PO_4^{3-}-P m^{-2} d^{-1} were achieved during the operating period from the middle of May to the end of November (200 days in a year). For horticultural facility (Proefcentrum voor Sierteelt vzw, Destelbergen, Belgium) that provided wastewater for this study, it has a working area of 400 m^2 and 800 L wastewater is produced per square meter per year with a nutrient concentration of 90 mg NO_3^--N L^{-1} and 15 mg PO_4^{3-}-P L^{-1}. Thus, to reduce the nitrogen and phosphorus concentrations to 10 mg TN L^{-1} and 1.0 mg TP L^{-1} respectively, it needs a working area of 640 m^2, which equals to 1.6 times of horticultural growth area.

4. Discussion

4.1 Temperature, irradiance, algal community and biomass production

Temperature has been reported as an important factor in the photosynthesis and growth of algal cells (Richmond, 2004). Generally, the algal growth rate increases with temperature to an optimum and then drops rapidly until lethal temperature is reached (Boelee et al., 2014). Furthermore, the tolerance of low or high temperature of algae is species-specific, for example, several species belonging to *Cladophora* had a proper temperature range of 0-18 °C, while for some others it was 26-29 °C (Breeman et al.,

2002). A study of Cambridge et al. (1991) showed that the 11 *Cladophora* species from Australia could only survive between 10 and 20 °C. In the study of Li et al. (2011), *Scenedesmus* showed no significant difference in the intrinsic growth rate when the temperatures varied from 10 to 30 °C, which indicated that it had a wide temperature tolerance. *Ulothrix zona* prefers low temperature and dominated in spring and autumn in the Milwaukee Harbor and the indoor experiment showed a low temperature and high irradiance was optimal for its net photosynthesis (Graham et al., 1985). In this study, *Scenedesmus* appeared and dominated over the whole experiment period of 2012 and 2013, which was in accordance with their wide tolerance of temperature in the literature.

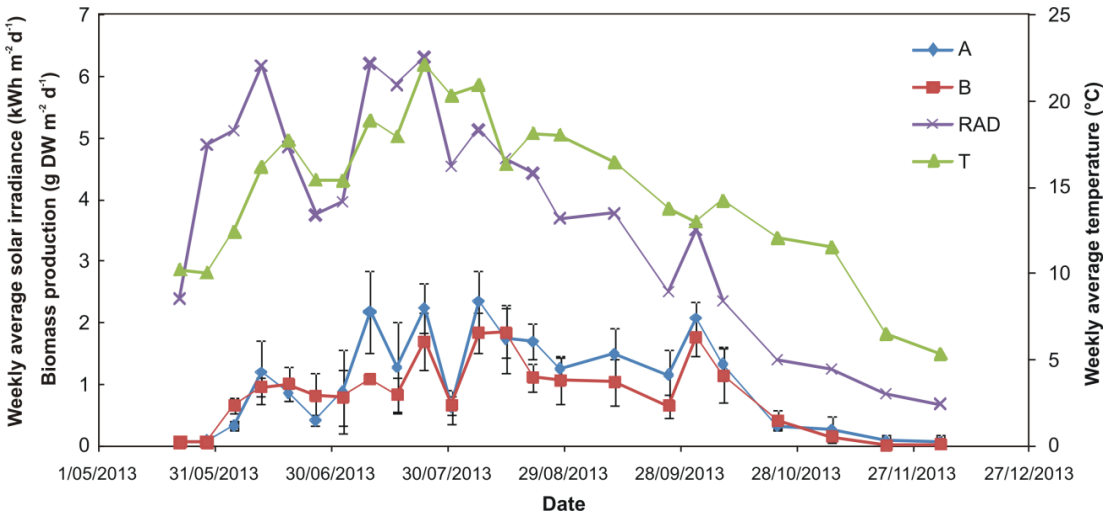

Fig. 4.8 The changes of biomass production from the ATS and temperature, solar irradiance in 2013 (A: inoculated with natural biofilms; B: inoculated with *Stigeoclonium* sp.; T: temperature; RAD: solar irradiance)

In the study of Sandefur et al. (2011), the monthly average biomass production of their ATS and the monthly average temperature showed a good linear correlationship ($R^2 = 0.65$, Fig. 4.9C). Similarly, in this study, the average biomass production of each week showed a similar pattern (Fig. 4.8) and the increasing temperature from 10 to 22 °C had positive effects on biomass production ($R^2 = 0.52$, 4.9A). However, the coefficient factor (0.12) of biomass production and the average temperature in this study was much lower than that of Sandefur et al. (1.86). Thus, there must be other factors limiting the biomass production other than temperature. The ATS of Sandefur et al. (2011) located in a subtropical area (Springdale, Arkansas, USA), which had an annual average sunhours of about 2800-3000 hours. In Ghent, Belgium where our ATS located, the weather is usually overcast and rainy, and the annual sunhours was only 1500-1600 hours with an annual sum irradiance of 1000-1100 kWh m^{-2} (KMI, http://www.meteo.be/). Moreover, the biomass production showed a similar pattern with

the solar irradiance and a correlationship (R^2 =0.5, Fig. 4.8, 4.9B). Therefore, the relatively low temperature and solar irradiance must have limiting effects on the biomass production of this ATS.

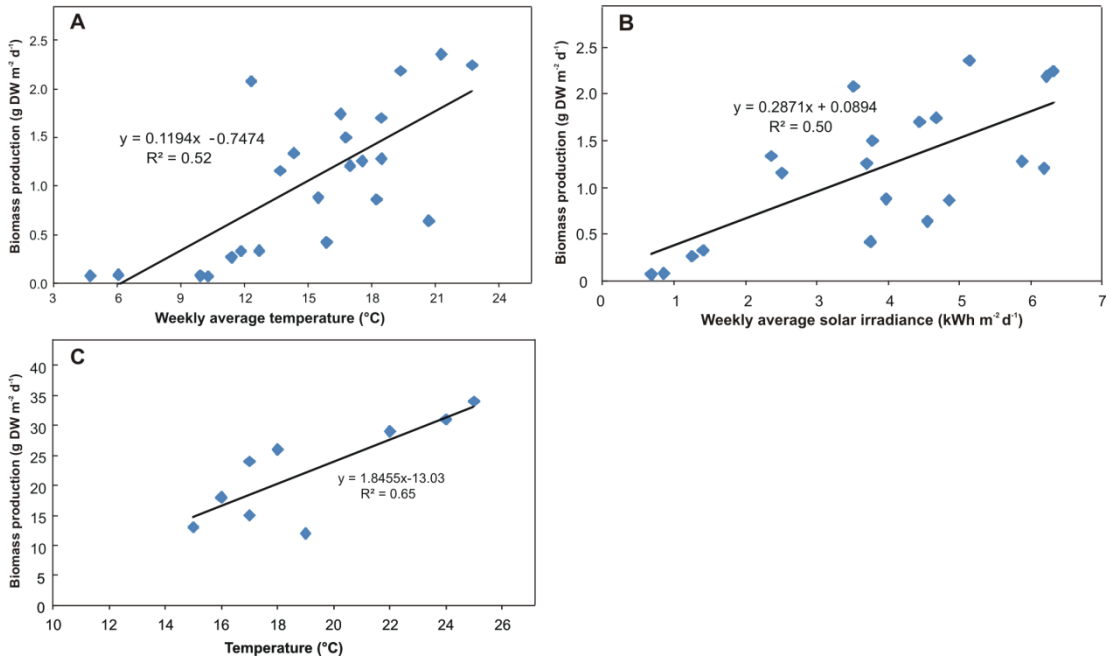

Fig. 4.9 A-B: The correlation between the weekly average temperature, solar irradiance and biomass production of the ATS in this study; C: The correlation between monthly average temperature and biomass production in Sandefur et al. (2011)

4.2 Priority effect, biodiversity and biomass production

The benthic algae *Stigeoclonium* had long-lasting priority effects on the periphyton community at a relatively low flow rate (Fig. 4.3A, B), but the dominance of *Stigeoclonium* showed no better performance in biomass production and nutrient removal than the ATS lanes inoculated with natural biofilm (Fig. 4.6, 4.7). Thus, the effect of periphyton community composition on biomass production was probably masked by other factors, such as light and temperature fluctuations as described above. Similarly, in 2013 the natural biofilm from a wastewater treatment plant and a monoculture of *Stigeoclonium* were used as the initial inoculum. Following the seven months running of the ATS, the lanes inoculated with the natural biofilm had a relatively higher biodiversity (1-2 genera more) than the lanes inoculated with *Stigeoclonium*, and a significantly higher biomass production (Fig. 4.8). It's widely assumed that a high diversity of the constructed community could enhance the function and biomass production (Cardinale, 2011; Ranalli & Lundholm, 2008). The algal community with high diversity can better grow in wastewaters because the loss of one species may be compensated by

others (Renuka et al., 2013), so the community can have a wide tolerance of external condition changes, such as temperature and irradiance fluctuations under outdoor conditions, or because of complementarity effects (Vanelslander et al., 2009). However, a high diversity with many planktonic microalgae may pose problems in harvesting and their resistance to predations.

4.3 Flow rate and algal community

In this study, from May to September of 2014, the flow rate was set at 8 L min^{-1} which was higher than the flow rate in 2012 and 2013. During this period, the biomass production of the ATS lanes with the same inoculums as in 2012 and 2013 was 1-3 g dry weight m^{-2} d^{-1} higher than in 2012 and 2013 (Fig. 4.5). The energy of the current velocity can drive the algal metabolism and chemical reactions, and the flowing water facilitates nutrient uptake by bringing metabolites to reaction sites and carrying away the waste. Therefore, a high current velocity can promote re-aeration of polluted waters through increased diffusion from the atmosphere and enhance the biomass production (Craggs et al., 1996).

In 2014, *Stigeoclonium* and *Pseudanabaena* were successfully inoculated to the ATS, but they lost their dominance in two weeks at a flow rate of 8 L min^{-1}. However, *Stigeoclonium* had a long-lasting dominance at a lower flow rate of 2 L min^{-1}. Generally, the current velocity determines the ability of the water to hold and transport suspended solids (Ahn et al., 2013; Craggs et al., 1996). As the benthic algae grow via attaching to the substrate using a holdfast or by producing mucilage (Sekar et al., 2004), the shear stress caused by the current can break the attachment. Thus, the benthic filamentous algae community is usually sensitive to shear stress and prefers a low water velocity (Ahn et al., 2013; Biggs & Thomsen, 1995; Dodds, 1991). However, the experiments with different flow rates were carried out in different seasons of 2014, so the effect of flow rate on the algal community, biomass production and nutrient removal should be further investigated during the same season.

4.4 Phosphorus removal and recovery

Generally, inorganic phosphorus can mainly be removed through three ways by periphyton: one mechanism was to be assimilated by algal cells; the second was the chemical precipitation with metal ions through the elevated pH caused by the photosynthesis of algae (de-Bashan & Bashan, 2004; Larsdotter et al., 2007; Roeselers et al., 2008) and the third way was surface adsorption onto periphyton via the formation of

hydrogen bonds between phosphate and polysaccharides of extracellular polymeric substances (EPS) (Li et al., 2013; Lu et al., 2014; Sheng et al., 2010; Wu et al., 2014). It was known that the phosphorus assimilation by algal cells was through active transport which was highly dependent on nitrogen availability of both algal cell tissues and the growth medium (Liu & Vyverman, 2015). In this study, the nitrogen removal rate was around 0.7-5.3 mg NO_3^--N L^{-1} d^{-1}, but the phosphorus removal rate reached as high as 4.5-5.0 mg PO_4^{3-}-P L^{-1} d^{-1}. One to two days after refreshing the wastewater, an increase of pH from 7.0 to 8.0-9.0 was observed, so chemical precipitation must have participated in phosphorus removal and the Ca^{2+} and Mg^{2+} concentrations of 100-160 and 20-40 mg L^{-1} enabled the precipitation in some extent.

Furthermore, based on the phosphorus reclaimed in the biomass and removed from the horticultural wastewater, a phosphorus recovery rate of 37-60% (Table 4.3) was detected. Therefore, it further indicated that other physical or chemical processes other than assimilation have participated in phosphorus removal (Kebede-Westhead et al., 2003; Lodi et al., 2003).

5. Conclusions

The outdoor ATS had a biomass production of 0.1-4.9 g dry weight m^{-2} d^{-1} and the high biodiversity improved the biomass production to an extent. *Stigeoclonium* had long-lasting priority effects on the periphyton community at a relatively low flow rate, but it showed no significant improvement in biomass production and nutrient removal. The low temperature and solar irradiance in Belgium had limiting effects on biomass production and nutrient removal of benthic algae community. *Scenedesmus* had a wide tolerance of temperature and appeared in large quantity during different seasons. Flow rate was a potential factor of biomass production and dominance of benthic filamentous algae in the periphyton community, but it needs further investigation in the same season.

Acknowledgements

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Chapter 5

Nutrient removal by the benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and their communities at varying flow rates on Algal Turf Scrubber

Liu, J., Danneels, B., Vanormelingen, P., Vyverman, W. Nutrient removal by the benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and their communities at varying flow rates on Algal Turf Scrubber, in preparation.

Abstract

Algal Turf Scrubber (ATS) is an attached cultivation system for biological wastewater treatment using benthic algae community. To assess the potential of benthic filamentous green algae in treating horticultural wastewater under natural conditions of Belgium, three strains and their mixture with naturally wastewater-born microalgae were cultivated in 250 ml indoor flasks as well as in 1 m^2 scale outdoor ATS with different flow rates. *Stigeoclonium* competed well with the natural wastewater growing microalgae and contributed to most of the biomass production both in indoor flasks and outdoor ATS at a relatively low flow rate of 2-6 L min^{-1} (water velocity 3-9 cm s^{-1}), while *Klebsormidium* was not suitable for growing in the horticultural wastewater under the tested conditions. Flow rate had a positive effect on biomass production and nitrogen removal of the algal community, while phosphorus removal was less influenced by flow rate probably due to chemical precipitation or surface adsorption.

Keywords: Algal Turf Scrubber, flow rate, horticultural wastewater, *Klebsormidium*, *Stigeoclonium*

1. Introduction

In the last half century the human activities have produced tremendous volumes of domestic, agricultural and industrial wastewater which has greatly increased the input of nutrients and other pollutants into natural water bodies (Abdel-Raouf et al., 2012; Boelee et al., 2014). One of the main problems of agricultural wastewater and the effluent of wastewater treatment systems is the remaining high concentration of inorganic nutrient, causing eutrophications when released into natural water ecosystems (de-Bashan & Bashan, 2004). Several studies have highlighted the potential of algae in removing inorganic nitrogen and phosphorus from domestic, agricultural and dairy wastewaters (Arbib et al., 2014; Mennaa et al., 2015; Van den Hende et al., 2014).

However, in the practical wastewater treatment, chemical composition of different wastewater sources vary greatly (Abdel-Raouf et al., 2012). For example, the wastewater produced from animal farms is usually rich in ammonium and organic nitrogen, while municipal wastewater has less nitrogen and phosphorus but more heavy metals than agricultural wastewater (Cai et al., 2013). Furthermore, each microalgae strain has its favorable growth conditions, such as pH, light, temperature, salinity, and preferred nitrogen type and N/P ratio (Besson & Guiraud, 2013; Cai et al., 2013; Lee et al., 2015; Liu & Vyverman, 2015; Markou & Georgakakis, 2011). In addition to the physiological factors, the interactions between the cultivated algae and other (micro) organisms, especially grazing by heterotrophic protists and small animals and competition with naturally occurring other microalgae in wastewater should be taken into consideration (Kesaano & Sims, 2014). For instance, filamentous algae with a large cell/colony size and indigestible cell wall are potentially more resistant to predation by grazers than the unicellular species (Guo et al., 2014; Wellnitz & Ward, 1998) and thus can improve the biomass production and nutrient removal efficiency (Kesaano & Sims, 2014). Besides removing nutrient, wastewater grown algae can also produce biomass for biofuel or high-value extracts (Mulbry et al., 2010; Zhu, 2015). For example, *Ulothrix* sp. contains a high amount of fatty acids C16:4ω3 and C18:3ω3 (Van den Hende et al., 2014). In our previous study (Chapter 2), *Klebsormidium* spp. and *Stigeoclonium* sp. have high C18:2ω6 and C18:3ω3 content respectively. Therefore, the selection of appropriate strains and a good understanding of the ecology of algal communities are critical in cleaning up a certain type of wastewater and producing valuable biomass (Roeselers et al., 2008).

Moreover, for the sustainable wastewater treatment using algae, it is required to get a high biomass recovery with a cost-efficient biomass harvesting method and discharge biomass-free effluent (Van den Hende et al., 2014). Benthic filamentous algae with large cell/colony size such as *Cladophora*, *Oedogonium* and *Spirogyra* have the ability to grow attached to substrates using a holdfast, and are easy, and thus cheap to harvest (Mulbry et al., 2010; Roberts et al., 2013; Sekar et al., 2004). Accordingly, several cultivation systems including Algal Turf Scrubber (ATS) and Rotating Algal Biofilm Reactor have been developed to make use of the above mentioned characteristics of benthic filamentous algae in wastewater treatment (Craggs et al., 1996; Kesaano & Sims, 2014).

ATS is a controlled ecosystem for wastewater treatment by flowing wastewater over an inclined surface which is covered by seeded periphyton biofilms. It has already been applied in treating polluted river water, agricultural and dairy manure wastewater with a growing area of 1 to 1000 m^2 (Adey et al., 2011; Craggs et al., 1996; Mulbry et al., 2008; Sandefur et al., 2011). The ATS is designed to promote biological wastewater treatment using benthic algae, by driving their photosynthesis to high levels, and harvesting the biomass periodically to remove the assimilated nutrient and stimulate further production (Adey et al., 2013; Sandefur et al., 2011). Accordingly, on an ATS flow rate is a vital factor of determining the ability of water to hold and transport suspended solids (Godillot et al., 2001). It can also facilitate nutrient uptake by bringing metabolites to reaction sites and carrying away the waste (Craggs et al., 1996). Zippel et al. (2007) reported that under the same light and temperature conditions, the biomass production was significantly higher at a flow rate of 100 L h^{-1} than at 25 L h^{-1}. However, the shear stress caused by water flow can break the attachment of benthic algae onto the supporting substrate and a weak shear stress or low flow rate/water velocity is usually preferred (Biggs & Thomsen, 1995; Larned, 2010; Sushchik et al., 2010). Therefore, flow rate could be a critical factor in determining algal community, biomass production and nutrient removal performance of an ATS.

In this study, *Klebsormidium* sp. and *Stigeoclonium* spp. were cultivated indoor individually and in mixture with the horticultural wastewater-born algae respectively. Then a mixture of them was inoculated to 1 m^2 outdoor ATS systems with different flow rates. The main objectives were to investigate: (1) the potential of *Klebsormidium* and *Stigeoclonium* in nutrient removal from horticultural wastewater and their competition with wastewater-born algae under controlled conditions; (2) the effect of flow rate on benthic algal community, biomass production and nutrient removal of an outdoor

ATS inoculated with *Klebsormidium* and *Stigeoclonium*. Moreover, the transferability of benthic filamentous algae in wastewater treatment from controlled laboratory conditions to natural outdoor conditions was evaluated.

2. Material and methods

2.1 Algal cultures

Three benthic filamentous green algae *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Stigeoclonium* sp. LJ2 used in this study were isolated as described previously (Liu & Vyverman, 2015). Subsequently, subcultures of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Stigeoclonium* sp. LJ2 for genetic analysis were harvested by centrifugation during exponential phase, and DNA was extracted following Zwart et al. (1998) using a bead-beating method with phenol extraction and ethanol precipitation. The primers NS7m and LR1850 (Friedl, 1996) were used for PCR amplification of ITS1, 5.8S and ITS2 regions of *Klebsormidium* sp. LJ2 and 18S rDNA of *Stigeoclonium* spp. was amplified with primers E2 from Van Hannen et al. (1999) and P4 from Moon-van der Staay et al. (2000). The PCR amplification conditions were: 5 min at 94 °C, 35 cycles of 2 min at 60 °C, 3 min at 68 °C and 15 min at 72 °C. The resulting PCR products were analyzed on an automated ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Waltham, USA). Sequence similarity searches were performed using a nucleotide BLAST search in GenBank, and the newly generated sequences were deposited in GenBank (accession numbers: KP165132, KR002183 and KR422334). All the strains were submitted to the BCCM/DCG culture collection (www.bccm.belspo.be, accession numbers: DCG0641, DCG0642 and DCG0643). Naturally occurring microalgae in the wastewater were collected by filtering the fresh horticultural wastewater through Whatman Grade 6 paper filters and were subsequently enriched in WC medium, but without vitamin addition or pH adjustment. *Klebsormidium* sp. LJ2, *Stigeoclonium* spp. and the natural wastewater microalgae were cultivated in WC medium under a 16:8 h light/dark cycle at a light intensity of 80-90 µmol photons m^{-2} s^{-1} at 23 °C in a climate room.

2.2 Indoor flasks

To investigate the potential of benthic filamentous algae in nutrient removal from horticultural wastewater, two different culture media were tested under indoor

conditions. First, the horticultural wastewater was collected from a horticultural company (Proefcentrum voor Sierteelt vzw) in Destelbergen, Belgium and filtered through Whatman Grade 6 paper filters and stored at 4 °C prior to the experiment. The second medium was a synthetic wastewater based on WC medium, to which additional NO_3^--N and PO_4^{3-}-P were added to a final concentration of 47.2 mg NO_3^--N L^{-1} and 11.6 mg PO_4^{3-}-P L^{-1} to match the nitrogen and phosphorus concentrations of the horticultural wastewater used in this study. The pH of the synthetic wastewater was adjusted to 7.0 (the same value as the horticultural wastewater) by adding 4% HCl after autoclaving.

Algal biomass from exponentially growing cultures (3 days after inoculation) of *Klebsormidium* sp. LJ2, *Stigeoclonium* LJ1, *Stigeoclonium* LJ2 and the enriched natural wastewater microalgae were filtered through a GF/F filter and washed with distilled water three times to remove any nutrient from the original medium. Next, 0.04 g of wet algal biomass from each of the four algal cultures was weighed and inoculated separately to 100 ml of the horticultural wastewater and synthetic wastewater respectively in 250 ml Erlenmeyer flasks in 9 replicates. A fifth inoculum consisted of a mixture of 0.01 g wet biomass of each of the four algal cultures. An additional subsample of the wet algal biomass was dried at 60 °C for 24 hours to measure its moisture content to determine their initial dry weight. Of the 9 replicate flasks, 3 were used for monitoring nutrient changes and harvested on day 9, 3 for harvesting on day 3 and 3 for harvesting on day 6 to get the growth curve (section 2.5). The cultivation conditions were the same as described previously (Liu & Vyverman, 2015) and Erlenmeyer flasks were manually shaken and replaced randomly on the shelf twice a day. The experiment lasted for 9 days by which time the concentration of NO_3^--N decreased below 10 mg L^{-1} (nitrogen discharge norm in Belgium) under most conditions.

2.3 Outdoor ATS

The ATS used in this study consisted of a water pump, a 120 L tank, a flow meter and a rectangular lane covered with plastic liner (a sketch drawing in Fig. 5.1). It was located under the natural conditions of Ghent, Belgium. The size of the lane was 0.39 m in width and 2.50 m in length (area 0.975 m^2), and it was set at a slope of 1%. Four units including three lanes each were set up, giving a total of 12 replicate lanes. The same horticultural wastewater as in section 2.2 was added to it. Then, 10 g wet biomass of a mixture of *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. from culture was inoculated to 12 individual lanes of the ATS. After a period of two weeks in which the algae could settle down, attach and form a biofilm, the bioreactor was started to run

with four different flow rates of 2, 4, 6 and 8 L min^{-1}(equal to water velocities of 2.8, 5.6, 8.4 and 11.2 cm s^{-1}), with triplicate lanes on three ATS units for each flow rate. After running for a week, the periphyton biomass was scrapped and discarded. From then on, the biomass was harvested weekly to monitor the algal community composition and biomass productivity. For each lane, the initial wastewater volume was 65 L, and distilled water was added regularly to compensate the evaporation. The wastewater was continuously recirculated. After each heavy rain, excess water was removed to 65 L level and the removed volume recorded. This experiment lasted from April to June 2015.

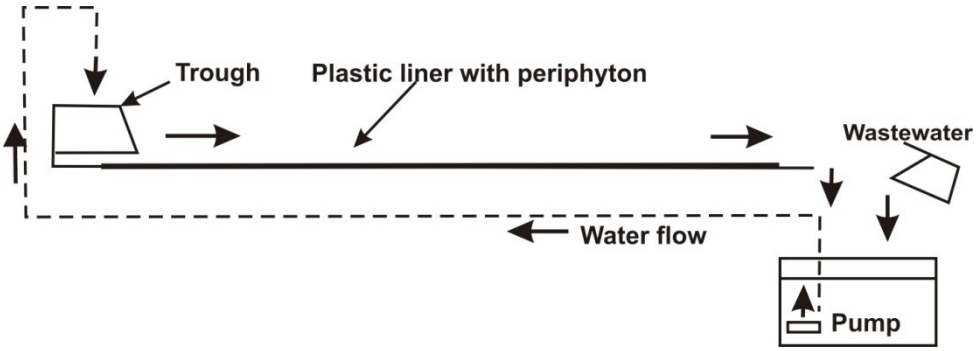

Fig. 5.1 Schematic drawing of the outdoor ATS used in this study

2.4 Wastewater chemical analysis

For the measurement of nutrient concentrations of the wastewater, 2 ml of wastewater was collected daily from the indoor flasks, while for the outdoor ATS 15 ml water was collected every two to three days after distilled water was added to the 65 L level. Fortunately, there was only one heavy rain during this operation period and no excess wastewater accumulated in the ATS tanks due to evaporation afterwards. The wastewater samples were filtered through Whatman grade 6 paper filters and the filtrate was used to measure pH, NO_3^--N and reactive phosphorus. pH was measured with a pH meter (PHM210, Unisense). NO_3^--N was measured with a spectrophotometer (Shimadzu UV-1601, Japan) at 220 and 275 nm following the ultraviolet spectrophotometric screening method (APHA, 1998). The reactive phosphorus was measured following the Vanadomolybdophosphoric acid colorimetric method with spectrophotometer at 400 nm (APHA, 1998).

2.5 Biomass harvesting and preparation

For the indoor flasks, the algal growth curve was determined by separately harvesting the biomass of three replicate flasks every three days and measuring the dry

weight (DW). Algal dry weight was determined by filtering the biomass through pre-weighed Whatman GF/F filters, freeze-drying overnight and weighing the dried filters with algal biomass the next day. The mean algal biomass production during the whole experiment period was calculated by Equation 5.1.

$$\text{Mean biomass production } (P_0, \text{ mg DW } L^{-1} d^{-1}) = \frac{DW_t - DW_0}{t * V} \qquad (5.1)$$

In Equation 5.1, DW_t represents the dry weight of the algae on day t, mg; DW_0 is the dry weight on day 0, mg; t is the cultivation time, d; V is the volume of the medium, L.

For the ATS, it was harvested every week by scraping the biomass from the plastic linear using a metal scraper and collected to a pre-weighed bottle, followed by centrifuging the biomass for 5 min at 2000 *g*. Then the wet biomass was weighed and a subsample was collected to an aluminum cup and weighed to measure its moisture content by drying for 24 hours to constant weight at 60 °C. The biomass productivity was calculated by Equation 5.2.

$$\text{Biomass productivity } (\text{g DW } m^{-2} d^{-1}) = \frac{(W_5 - W_3)(W_2 - W_1)}{(W_4 - W_3) * A * N} \qquad (5.2)$$

In Equation 5.2, W_1 was the weight of the bottle, g; W_2 was the weight of bottle and wet biomass, g; W_3 was the weight of aluminum cup, g; W_4 was the weight of aluminum cup and wet biomass, g; W_5 was the weight of aluminum cup and dry biomass, g; A was the area of the bioreactor, m²; N was the sampling interval, day.

Nitrogen and phosphorus content of the harvested algal biomass were determined with the methods described previously (Liu & Vyverman, 2015). Nitrogen and phosphorus recovery rate in the biomass was calculated with Equation 5.3.

$$\text{N (P) recovery rate } (\%) = \frac{M * C_{N(P)}}{V * (C_0 - C_1)} * 100\% \qquad (5.3)$$

In Equation 5.3, M was the biomass produced between day t_0 and t_1, mg; $C_{N(P)}$ was nitrogen or phosphorus content of the biomass, % of DW; V was the volume of the wastewater, L; C_0 and C_1 were the nitrogen or phosphorus concentrations on day t_0 and t_1, mg N(P) L^{-1}.

2.6 Algal community structure

Subsamples for algal community analysis were collected on day 0 and 9 for the indoor flasks and every week for the ATS, and fixed with an equal volume of 4% for-

malin. To assess the algal composition of the biofilms, 50 µl of the sample was transferred to a microscope slide and 9 photos were randomly taken at 400× magnifications (Leitz Diaplan Microscope, Germany), and this was done in triplicates. Then the cell numbers of each species on each photo were counted with Image J and converted to the biomass contribution by multiplying with their biovolume, which was calculated with the equations proposed by Hillebrand et al. (1999) by measuring the sizes of 30-40 cells with Image J. Both *Stigeoclonium* strains were counted as *Stigeoclonium*.

2.7 Statistical analysis

Two-way ANOVAs were used to test for statistical differences in biomass production or nutrient content of the biomass with algal inoculum and growth medium as independent fixed factors (using STATISTICA 7.0). Post-hoc Tukey tests were used to determine significant pairwise differences. One-way ANOVA was used to test for statistical differences in biomass production or nutrient content of the biomass from the outdoor experiment with flow rate as independent fixed factor. A significance level of $p < 0.05$ was applied throughout.

3. Results and discussion

3.1 Algal composition and biomass production

3.1.1 Indoor flasks

The naturally occurring wastewater microalgae were dominated by *Chlamydomonas* sp. (7%), *Chlorella* sp. (67%) and various filamentous cyanobacteria (25%). After 9 days cultivation, the filamentous algae *Stigeoclonium* spp. competed well with the natural wastewater algae and the algal composition was relatively stable in both of horticultural and synthetic wastewater with a proportion of 62% on day 0 and 69% and 68% on day 9 respectively (Fig. 5.2), while *Klebsormidium* sp. LJ2 decreased from 26% to 13% and 11% in horticultural and synthetic wastewater respectively. For the natural wastewater algae, *Chlorella* sp. grew well and its relative abundance increased from 67% to 75% in the horticultural wastewater and 90% in the synthetic wastewater (Fig. 5.2), while cyanobacteria decreased from 25% on day 0 to 14% and 1% on day 9 in the horticultural and synthetic wastewater respectively.

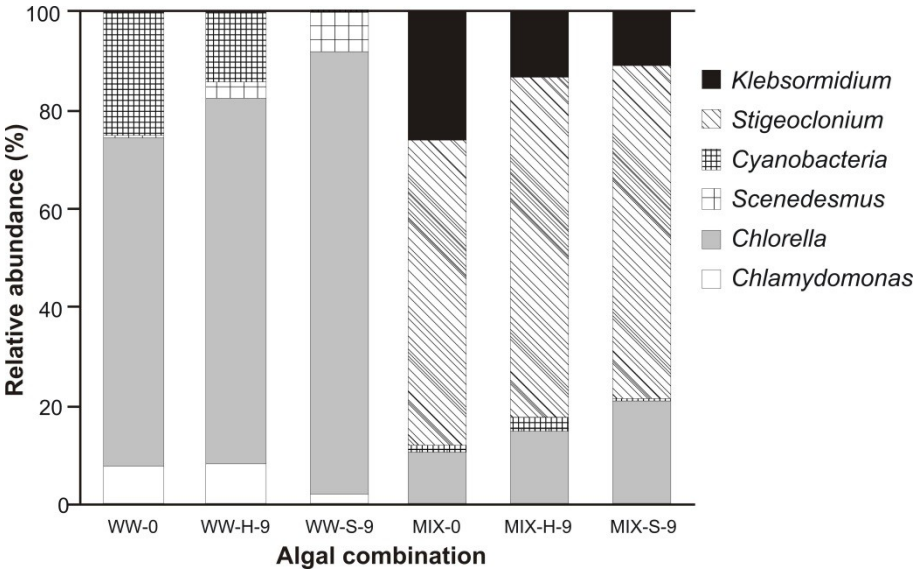

Fig. 5.2 Algal community composition of natural wastewater algae (WW) and the mixture of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and natural wastewater algae (MIX) in synthetic (S) or horticultural (H) wastewater on day 0 (0) and after nine days (9)

As shown in Fig. 5.3A and 5.3B, the monocultures of *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. had higher biomass production in the synthetic wastewater (36.0-63.3 mg L^{-1} d^{-1}) than in horticultural wastewater (13.5-50.8 mg L^{-1} d^{-1}). In contrast, the natural occurring wastewater algae and the mixture had higher biomass production in horticultural wastewater (53.7-57.1 mg L^{-1} d^{-1}) than in synthetic wastewater (46.5-49.0 mg L^{-1} d^{-1}). The two-way ANOVA showed that there was a significant difference in the biomass production between the five algae inoculums ($F = 166.2$, $p < 0.001$) and also between the two media ($F = 41.3$, $p < 0.001$). Also the interaction of algae inoculums and wastewater media had a significant effect on the biomass production ($F = 40.1$, $p < 0.001$). The Post-hoc Tukey test indicated that the inoculum effect on biomass production was mainly caused by the lower biomass production of *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 than the others, and the medium effect was mainly due to the lower biomass production of the monocultures in horticultural wastewater than in synthetic wastewater.

The monocultures of *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 had significantly lower biomass production (all $p < 0.001$) in the horticultural wastewater than *Stigeoclonium* sp. LJ2, the natural wastewater algae and the mixture. Thus, *Stigeoclonium* sp. LJ2 was more suitable for growing in the horticultural wastewater than *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1. Furthermore, the algal combi-

nation with a high diversity can adapt to various kinds of wastewater and enhance the biomass productivity. It was in accordance with the report of Arbib et al. (2014) that a natural bloom had higher biomass production in the treated urban wastewater than monoculture of *Chlorella vulgaris* and *Chlorella kessleri* and the work of Silva-Benavides and Torzillo (2012) that the co-culture of *Chlorella vulgaris* and *Planktothrix isothrix* had higher biomass production than the monoculture of either *Chlorella vulgaris* or *Planktothrix isothrix* in municipal wastewater.

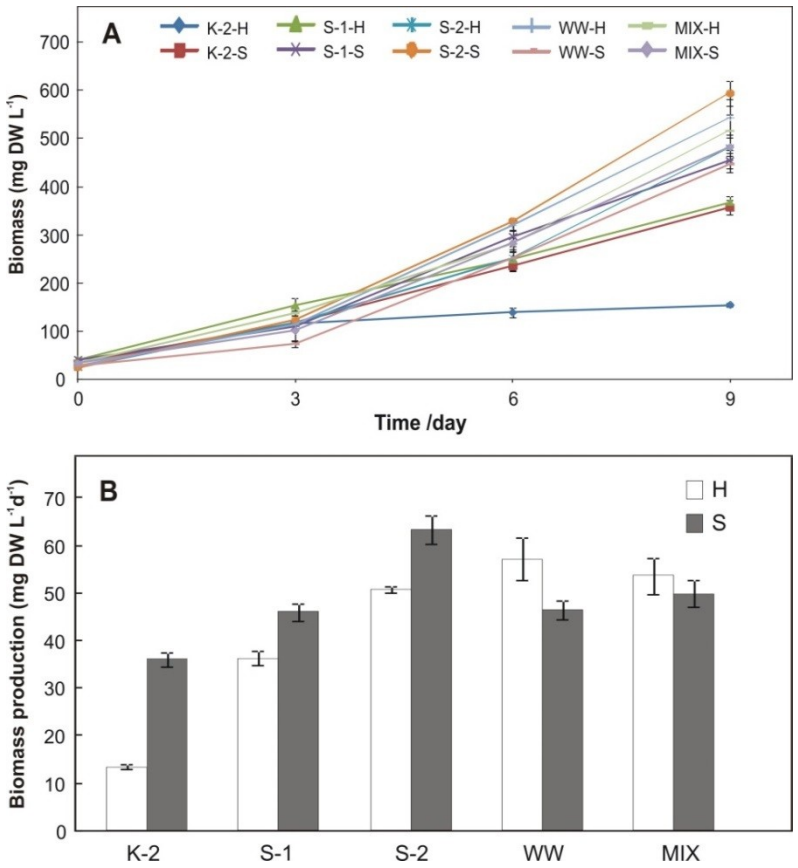

Fig. 5.3 A: Average biomass (mg DW L^{-1}) accumulated over time of *Klebsormidium* sp. LJ2 (K-2), *Stigeoclonium* sp. LJ1 (S-1), *Stigeoclonium* sp. LJ2 (S-2), natural wastewater algae (WW) and mixture (MIX) respectively in indoor flasks with horticultural (H) and synthetic wastewater (S); B: Average biomass production (mg DW L^{-1} d^{-1}) of the five algal combinations in indoor flasks with horticultural and synthetic wastewater.

3.1.2 Outdoor ATS

Triplicate ATS lanes (Fig. 5.1) were operated at flow rates of 2-8 L min^{-1} from May to June 2015. As shown in Fig. 5.3, *Klebsormidium* sp. LJ2 only had a low abundance (less than 4%) in the first two weeks of the experiment and then disappeared. For *Stigeoclonium* spp., it had an abundance of 66-73% in the beginning and then its abun-

dance at the flow rate of 8 L min^{-1} decreased sharply to 8% by week 5 (Fig. 5.3D), while its abundance remained 46% at flow rates of 2 L min^{-1} and 20-31% at 4-6 L min^{-1} by week 5 (Fig. 5.4A, B and C). For the unicellular algae, *Chlamydomonas*, *Desmodesmus* and *Scenedesmus* were the main genera and showed a gradually increase in their relative abundance. Especially at the flow rate of 8 L min^{-1}, the abundance of *Desmodesmus* greatly increased from 5% in the beginning to 71% by week 5 (Fig. 5.3D). In summary, the benthic filamentous algae were easily influenced by flow rate and the results of this study were in accordance with the reports of Ahn et al. (2013) and Dodds (1991).

The biomass production showed an increasing trend following the experiment going on from May to June (Fig. 5.5), and one-way ANOVA indicated that there was a significant difference in biomass production between the tested four flow rates ($F = 4.0$, $p = 0.016$). Specifically, the lowest biomass production (0.8-1.7 g DW m^{-2} d^{-1}) was observed at the lowest flow rate of 2 L min^{-1}, while the highest (1.2-2.0 g DW m^{-2} d^{-1}) was produced at the flow rate of 8 L min^{-1}. Moreover, the biomass produced showed a large standard deviation between the triplicates. That was due to the lower biomass production of the middle lane than the other two lanes of the same ATS unit.

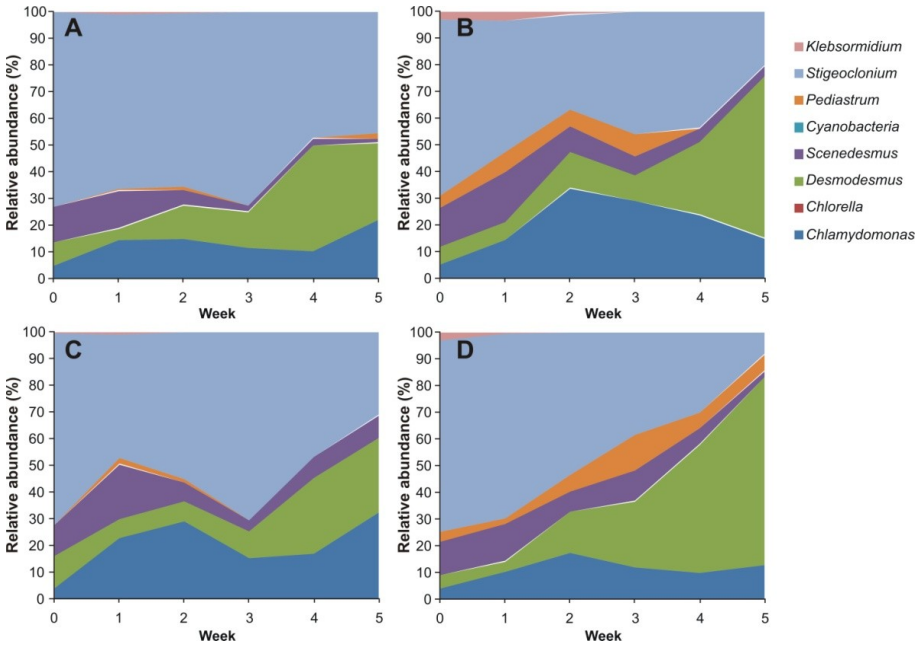

Fig. 5.4 A-D: Algal community composition changes over time of the outdoor ATS under flow rates of 2, 4, 6 and 8 L min^{-1} respectively.

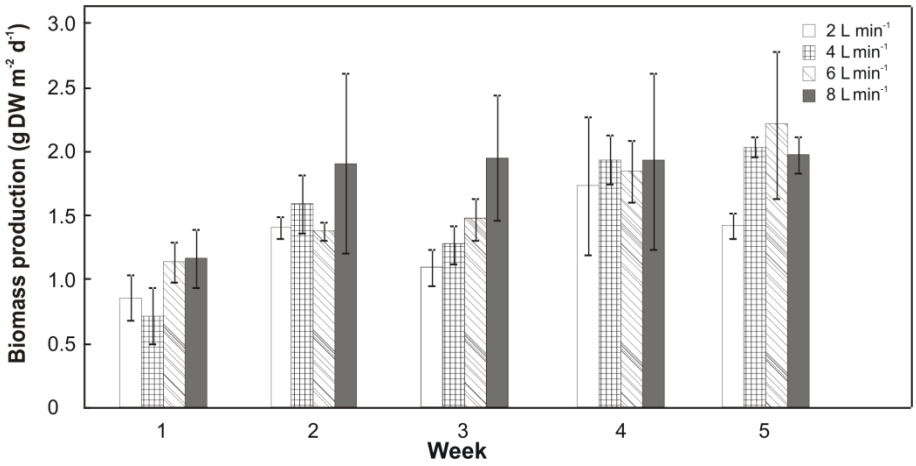

Fig. 5.5 The biomass production on the outdoor Algal Turf Scrubber (g DW m^{-2} d^{-1}) under flow rates of 2-8 L min^{-1}. The error bars correspond to the standard deviation of triplicates.

3.2 Nitrogen and phosphorus removal

3.2.1 Indoor flasks

For the experiment studying the nutrient removal by different algal combinations from the horticultural and synthetic wastewater (Fig. 5.6A, B), the monoculture of *Stigeoclonium* sp. LJ2 had the highest daily nitrogen removal rate (Table 5.1, maximally 8.0 and 8.6 NO_3^--N mg L^{-1} d^{-1} in the horticultural and the synthetic wastewater respectively), while the monoculture of *Klebsormidium* sp. LJ2 had the lowest nitrogen removal rate (maximally 4.5 and 5.7 mg NO_3^--N L^{-1} d^{-1}). In terms of phosphorus removal, the monoculture of *Stigeoclonium* sp. LJ2 had the highest daily phosphorus removal rate (maximally 5.4 mg PO_4^{3-}-P L^{-1} d^{-1}) in the horticultural wastewater, while the natural wastewater algae had the lowest (maximally 3.8 mg PO_4^{3-}-P L^{-1} d^{-1}).

After 9 days cultivation, the nitrogen removal efficiency of the synthetic wastewater (59-99%) was slightly higher than the horticultural wastewater (20-86%) for the five algal combinations (Table 5.1, Fig. 5.6A). From Fig. 5.6A, it can be concluded that nitrogen was removed at a higher rate from synthetic wastewater than from horticultural wastewater by all five algal combinations, especially the monoculture of *Klebsormidium* sp. LJ2 and both *Stigeoclonium* strains. For example, the NO_3^--N concentration of horticultural wastewater treated by *Klebsormidium* sp. LJ2 remained around 40 mg L^{-1} from day 2, while it was reduced to 20 mg L^{-1} in synthetic wastewater by day 9. Moreover, the NO_3^--N removal process from horticultural and synthetic

wastewater by the natural wastewater algae was quite similar (Table 5.1, Fig. 5.6A). It indicated that the natural wastewater algae were well adapted to the horticultural wastewater and could efficiently assimilate nitrogen from the wastewater. The monoculture of *Stigeoclonium* sp. LJ2 showed higher nitrogen removal efficiency from both horticultural and synthetic wastewater than *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1. It indicated that *Stigeoclonium* sp. LJ2 had a good capacity of assimilating nitrogen from the horticultural wastewater.

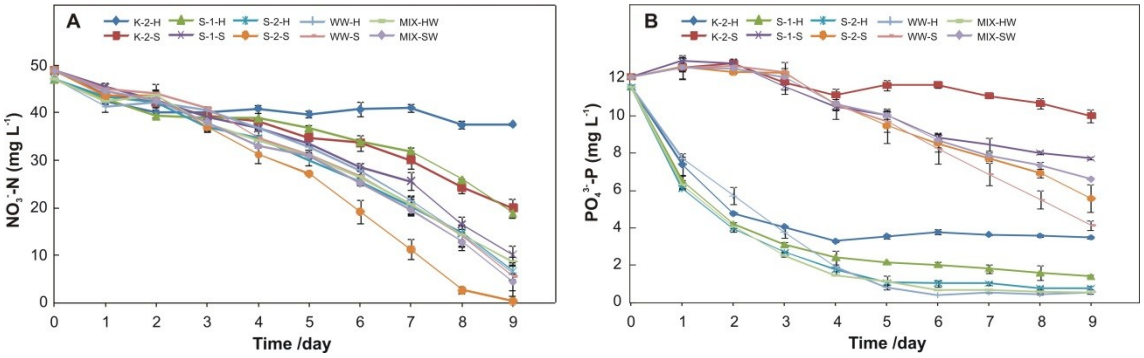

Fig. 5.6 A, B: NO_3^--N and PO_4^{3-}-P concentration (mg L^{-1}) changes process of *Klebsormidium* sp. LJ2 (K-2), *Stigeoclonium* sp. LJ1 (S-1), *Stigeoclonium* sp. LJ2 (S-2), natural wastewater algae (WW) and mixture (MIX) respectively in horticultural (H) and synthetic wastewater (S) in indoor flasks over time. The error bars correspond to the standard deviation.

For phosphorus removal, there was a sharp decrease in the horticultural wastewater after the first day, during which the PO_4^{3-}-P concentration decreased from 11.6 mg L^{-1} to 6.1-7.7 mg L^{-1}. Accordingly, it resulted in a maximal phosphorus removal rate of 4.1, 5.0, 5.4, 3.8 and 5.2 mg PO_4^{3-}-P L^{-1} d^{-1} for the monoculture of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2, natural wastewater algae and the mixture respectively. Compared to the phosphorus removal process of horticultural wastewater, both the phosphorus removal rate and efficiency from synthetic wastewater were lower for these five algal combinations (Fig. 5.6B and Table 5.1). Their maximal phosphorus removal rates were 1.0, 1.3, 1.6, 1.8 and 1.3 mg PO_4^{3-}-P L^{-1} d^{-1} for the monoculture of *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2, natural wastewater algae and the mixture respectively.

The big difference in the maximal phosphorus removal rate between the horticultural and synthetic wastewater could be caused by the chemical precipitation of phosphorus. Chemical precipitation has been documented as an important phosphorus removal mechanism from the wastewater by algae (de-Bashan & Bashan, 2004). The

Table 5.1 Maximal NO_3^--N and PO_4^{3-}-P removal rate ($R_{max, N}$, $R_{max, P}$, mg N or P L^{-1} d^{-1}), the final nitrogen and phosphorus removal efficiency (RE_N, RE_P, %), nitrogen and phosphorus content and recovery rate of *Klebsormidium* sp. LJ2 (K-2), *Stigeoclonium* sp. LJ1 (S-1), *Stigeoclonium* sp. LJ2 (S-2), natural wastewater algae (WW) and mixture of the above four (MIX) in the horticultural (H) and synthetic wastewater (S) under indoor conditions

Algal combination	K-2		S-1		S-2		WW		MIX	
	H	S	H	S	H	S	H	S	H	S
$R_{max, N}$ (mg N L^{-1} d^{-1})	4.7	5.7	6.0	9.1	8.0	8.6	7.8	8.5	6.4	8.3
RE_N (%)	20	59	60	79	86	> 99	86	89	82	91
N content (%)	3.3 ± 0.1	5.4 ± 0.1	4.7 ± 0.1	5.4 ± 0.2	5.6 ± 0.2	5.6 ± 0.3	5.2 ± 0.3	6.4 ± 0.1	5.2 ± 0.1	6.3 ± 0.1
N recovery (%)	53 ± 1	67 ± 1	61 ± 1	63 ± 1	66 ± 1	69 ± 0.1	69 ± 1	67 ± 3	69 ± 6	66 ± 3
$R_{max, P}$ (mg P L^{-1} d^{-1})	4.1	1.0	5.0	1.3	5.4	1.6	3.8	1.8	5.2	1.3
RE_P (%)	70	17	88	36	93	54	95	66	95	45
P content (%)	2.5 ± 0.1	0.7 ± 0.02	2.2 ± 0.1	1.2 ± 0.04	1.8 ± 0.1	1.2 ± 0.03	1.6 ± 0.09	1.4 ± 0.1	1.5 ± 0.05	1.3 ± 0.01
P recovery (%)	46 ± 6	93 ± 7	78 ± 3	96 ± 1	81 ± 5	90 ± 0.1	79 ± 11	72 ± 4	69 ± 7	99 ± 1

pH of the wastewater can be prompted by the consumption of CO_2 through the algal photosynthesis and then result in the phosphorus precipitation (Craggs et al., 1996; Larsdotter et al., 2010; Roeselers et al., 2008). In this study, the pH increased greatly from 7.0 on day 0 to 8.5 on day 1 and then above 9.0 afterwards in both the horticultural and synthetic wastewater. However, in the synthetic wastewater, the chelating agent EDTA which can prevent phosphorus precipitation (de-Bashan & Bashan, 2004) was present and caused a lower phosphorus removal rate and thus a lower phosphorus removal efficiency than horticultural wastewater.

3.2.2 Outdoor ATS

For the outdoor ATS with different flow rates, the lanes under a flow rate of 8 L min^{-1} had the highest nitrogen removal rate and efficiency while the lanes at 2 L min^{-1} had the lowest (Fig. 5.7A, Table 5.2). In terms of phosphorus removal, the lanes under the flow rate of 8 L min^{-1} had slightly higher phosphorus removal efficiency from day 2 on while the other three showed no difference (Fig. 5.7B).

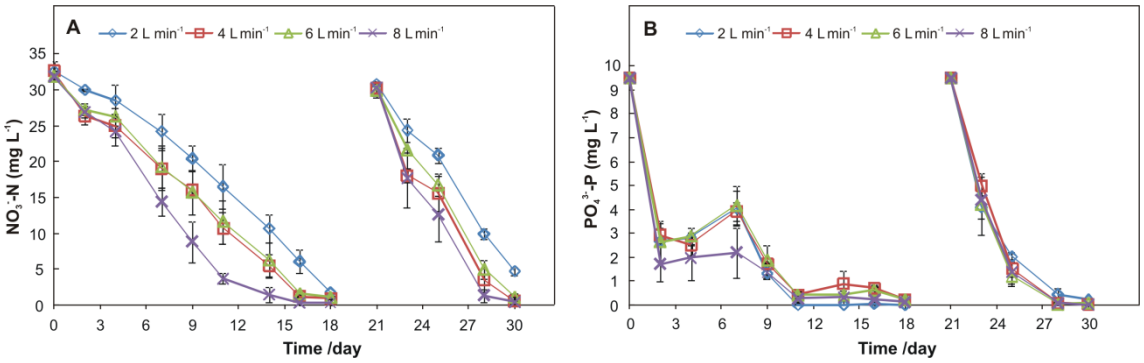

Fig. 5.7 A, B: NO_3^--N and PO_4^{3-}-P concentration (mg L^{-1}) changes process of the outdoor ATS at flow rates of 2-8 L min^{-1} over time.

As shown in Fig. 5.7A, the NO_3^--N removal process accelerated following the running of the ATS, and it decreased from 18 to 9 days to remove NO_3^--N from 32 mg L^{-1} to 0. This was in accordance with the biomass accumulation process (Fig. 5.5). Generally, the nitrogen removal rate at a flow rate of 8 L min^{-1} was higher than at 2-6 L min^{-1} and it was maximally 6.4 mg L^{-1} d^{-1} (Table 5.2), while it was 3.5-6.1 mg L^{-1} d^{-1} at flow rates of 2-6 L min^{-1}. For the ATS lanes with flow rate of 8 L min^{-1}, it took 9 days during the first cycle to reach the nitrogen discharge norm in Belgium (10 mg TN L^{-1}), while it took 14, 11 and 12 days for the lanes with flow rates of 2, 4 and 6 L min^{-1} respectively.

Table 5.2 Maximal NO_3^--N and PO_4^{3-}-P removal rate ($R_{max, N}$, $R_{max, P}$, mg N or P L^{-1} d^{-1}), nitrogen and phosphorus removal efficiency (RE_N, RE_P, %), nitrogen and phosphorus content of harvested biomass and their recovery rate at flow rates of 2-8 L $min^{-1}$of outdoor ATS

Flow rate (L min^{-1})	2	4	6	8
$R_{max, N}$ (mg N L^{-1} d^{-1})	3.5	6.1	5.9	6.4
RE_N (%)	88 ± 5	98 ± 1	96 ± 1	99 ± 1
N content (%)	6.2 ± 0.8	6.3 ± 0.3	6.8 ± 0.8	6.5 ± 0.3
N recovery (%)	86 ± 7	83 ± 12	73 ± 18	73 ± 18
$R_{max, P}$ (mg P L^{-1} d^{-1})	3.4	3.3	3.5	3.9
RE_P (%)	> 99	> 99	> 99	> 99
P content (%)	2.1 ± 0.5	2.1 ± 0.4	2.3 ± 0.8	2.3 ± 0.6
P recovery (%)	60 ± 5	72 ± 14	79 ± 12	86 ± 21

In terms of the phosphorus removal, there was a sharp decrease in PO_4^{3-}-P concentration from 9.5 to 1.7-2.9 mg L^{-1} for all the lanes after running two days, which was similar to the phosphorus removal process of the indoor experiment. Generally, phosphorus can also be removed through surface adsorption by periphyton besides assimilation and chemical precipitation as described above (Lu et al., 2014; Sañudo-Wilhelmy et al., 2004). It was known that the phosphorus assimilation by algal cells was through active transport which was highly related to the nitrogen availability of both algal cell tissues and the medium (Liu & Vyverman, 2015). In this study, the nitrogen removal rate was around 1-3 mg L^{-1} d^{-1}, but the phosphorus removal rate reached to 3.4-3.9 mg L^{-1} d^{-1}, most probably other mechanisms including chemical precipitation and surface adsorption were functioning in phosphorus removal during the first few days. Therefore, because of the participation of precipitation or adsorption, the effect of flow rate on phosphorus removal was suppressed.

3.3 Nutrient composition and recovery by biomass

For the indoor experiment, nitrogen and phosphorus content of the biomass were 3.3-6.3% and 0.7-2.5% of dry weight respectively (Table 5.1). Two-way ANOVA showed that there was significant difference in nitrogen content between the five algal combinations ($F = 85.0$, $p < 0.001$) and between the two wastewater media ($F = 274.7$, $p < 0.001$), and the interaction between algal combination and the wastewater media was also highly significant ($F = 32.9$, $p < 0.001$). The Post-hoc Tukey test indicated that

the effect of algae inoculum on nitrogen content was mainly caused by the lower or higher nitrogen content of *Klebsormidium* sp. LJ2 and natural wastewater microalgae than the others. The effect of wastewater media was due to the lower nitrogen content in horticultural wastewater than in synthetic wastewater.

Similarly, the two-way ANOVA also indicated there was significant difference in phosphorus content between the five algae inoculums ($F = 9.5$, $p < 0.001$) and between the two types of wastewater ($F = 522.9$, $p < 0.001$), and the interaction between algae inoculums and the wastewater media was highly significant as well ($F = 87.3$, $p < 0.001$). The Post-hoc Tukey test indicated that the effect of species on phosphorus content was mainly caused by the higher phosphorus content of *Klebsormidium* sp. LJ2 and *Stigeoclonium* sp. LJ1 than the others, and the effect of wastewater media was caused by the higher phosphorus content in horticultural wastewater than in synthetic wastewater.

Nitrogen recovery rate by algal biomass of these five algae inoculums varied between 53% and 69%, while it was 46-99% for phosphorus (Table 5.1). Although the biomass produced from horticultural wastewater had higher phosphorus content than from synthetic wastewater, the phosphorus recovery rate of *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. and the mixture was higher in synthetic wastewater (90-99%) than in horticultural wastewater (46-81%). This further indicated that other process than assimilation participated in phosphorus removal from the horticultural wastewater.

Compared to the indoor flasks, the nitrogen and phosphorus content of the biomass harvested from the outdoor ATS was more sensitive to nitrogen and phosphorus concentrations of the wastewater and it varied between 4.9% and 6.9%, 1.5% and 3.6% for nitrogen and phosphorus respectively (Table 5.2). It showed no significant difference in biofilm nitrogen and phosphorus content between the tested four flow rates ($F = 2.1$, $p = 0.184$; $F = 1.8$, $p = 0.23$ for nitrogen and phosphorus respectively). However, phosphorus recovery rate of the ATS increased greatly from 60 ± 5% to 86 ± 21% following the increase of flow rate from 2 to 8 L min^{-1}. That was most likely because of higher biomass production at higher flow rate, which produced more periphyton biomass to absorb and/or adsorb phosphorus from wastewater (Liu & Vyverman, 2015; Lu et al., 2014).

3.4 Potential of benthic filamentous algae in outdoor bioreactor

In this study, both *Stigeoclonium* strains grew well both in monoculture and mixture with the natural wastewater algae in horticultural wastewater and efficiently assimilated nitrogen and phosphorus from wastewater under controlled indoor conditions. In the outdoor ATS, they were successfully inoculated and maintained their dominance with a contribution of 46-73% to the total biomass at a low flow rate of 2 L min^{-1} (water velocity of 3 cm s^{-1}), while they gradually lost their dominance at high flow rates. *Klebsormidium* sp. LJ2 had a much lower biomass and nutrient removal efficiency in the horticultural wastewater than *Stigeoclonium* spp. in the indoor experiment. Similarly, *Klebsormidium* sp. LJ2 only had a relative abundance of less than 3.5% in the beginning few weeks on the outdoor ATS. That was likely due to the inhibiting effect of horticultural wastewater or its relatively low growth rate and nutrient uptake capability as shown in the synthetic wastewater and in our previous study (Liu & Vyverman, 2015).

Flow rate or water velocity was proved to be a critical factor in algal community, biomass production and nutrient removal efficiency of ATS in this study. Specifically, a low flow rate enhanced the dominance of benthic filamentous algae of the periphyton biofilm, while a high flow rate promoted the mass exchange and consequently the algal biomass accumulation and nutrient removal efficiency. These findings of this study were in accordance with the previous reports (Ahn et al., 2013; Craggs et al., 1996; Dodds, 1991; Zippel et al., 2007). Therefore, selection of appropriate algal species and optimization of flow rate for a proper algal community and efficient nutrient removal should be taken into consideration for the future work on benthic filamentous algae based bioreactors.

4. Conclusions

Stigeoclonium performed well in growth and removing nutrient from the horticultural wastewater both in indoor flasks and outdoor ATS and can be a good candidate for treating horticultural wastewater, while the *Klebsormidium* strain was not suitable. On the ATS with low flow rate, *Stigeoclonium* could compete with the naturally occurring unicellular microalgae and dominated on the bioreactor. Hihg flow rate had significantly positive effect on the biomass production and nitrogen removal rate of the outdoor ATS, while phosphorus removal was less influenced, probably due to chemical precipitation and/or surface adsorption. This study provided an attempt to select the

appropriate benthic algae and optimize the flow rate to improve biomass production and nutrient removal efficiency of an outdoor ATS.

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Chapter 6

General discussion and future perspectives

1. Introduction

This work aimed to contribute to the understanding of growth, biomass production, nutrient uptake capacity and biochemical composition of benthic filamentous algae and their communities. To this end, we carried out complementary experiments in indoor flasks and outdoor Algal Turf Scrubber (ATS). Various nitrogen and phosphorus conditions and light deprivation were set under indoor conditions to elucidate the differences in algal growth, nutrient uptake capacity and biochemical composition of several attached filamentous algae isolated from a pilot ATS (Chapters 2 and 3). To investigate their performance in nutrient removal and the interaction with other microorganisms, the benthic algae were inoculated individually and in mixture to horticultural wastewater under both laboratory controlled and outdoor natural conditions with different flow rates (Chapters 4 and 5). These approaches proved to be valuable in obtaining insights in several aspects of the characteristics of attached filamentous algae, being (i) differences in biochemical composition (N, P and protein content, fatty acid profile) and nutrient uptake capacity (Chapters 2 and 3), (ii) priority effects lasting of benthic filamentous algae on algal community composition and nutrient removal on ATS (Chapter 4), (iii) the relationship between flow rate and the dominance of filamentous green algae (Chapter 5).

2. Main outcomes and positioning of this work

2.1 Species selection for large-scale cultivation and application

It is well-known that different algal groups differ greatly in growth rate, biochemical composition and thus nutrient requirement for growth (Geider & La Roche, 2002; Ho et al., 2003; Li et al., 2010; Wu et al., 2013). As shown in Chapter 3 of this study, the benthic algae *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. had different optimal N/P ratios and thus different nitrogen and phosphorus requirements for their growth. Different algal species also vary significantly in growth rate and biochemical composition including nitrogen, phosphorus and total protein content and fatty acid profile in response to changes in N/P conditions, growth phases and light conditions (Chapters 2 and 3). Accordingly, *Klebsormidium* spp., *Stigeoclonium* sp. LJ1 and *Uronema* sp. can be good potential sources of producing C18:2ω6, C18:3ω3 and protein respectively. Furthermore, their nutrient uptake capacity varied greatly as well, with *Stigeoclonium* sp. LJ2 having the

highest phosphorus uptake capacity and *Pseudanabaena* sp. having the highest nitrogen uptake capacity of the studies species.

Additionally, the responses of periphytic algae to external environmental changes were investigated in outdoor ATS, involving variability in temperature, flow rate and interaction with other microorganisms (Chapters 4 and 5). *Stigeoclonium* grew well in the horticultural wastewater under indoor conditions and also maintained its dominance through priority effects on the algal community at a relatively low flow rate and achieved a high nitrogen and phosphorus removal efficiency on the outdoor ATS. *Klebsormidium* sp. LJ2 grew relatively slow in the horticultural wastewater indoors compared to artificial medium and the other algae tested and in parallel failed to dominate the periphyton community under outdoor conditions. *Pseudanabaena* sp. had the highest growth rate and nitrogen uptake rate under indoor conditions (Chapter 3), but it lost its dominance gradually to *Stigeoclonium* spp. in the outdoor ATS, probably due to its lower nitrogen uptake capacity than *Stigeoclonium* at low nitrogen concentrations as elucidated in Chapter 3.

Therefore, we conclude that the benthic alga *Stigeoclonium* is robust under outdoor conditions and suitable for nutrient removal from horticultural wastewater in an attached cultivation system at a relatively low flow rate. Moreover, larger screenings of additional filamentous algae strains will contribute greatly to selecting more robust and appropriate strains for wastewater treatment.

2.2 Biodiversity and biomass production of benthic algal community

The notion that a combination of more than one microorganism performs better than a single organism in terms of productivity or other ecosystem functions is a well-known phenomenon in ecology (Brenner et al., 2008; Vanelslander et al., 2009), and currently gains broader attention in algae mass cultivation (Renuka et al., 2013; Silva-Benavides & Torzillo, 2012). In Chapter 4, a natural biofilm and culture of *Stigeoclonium* were used as the initial inoculums in 2013. During the seven months experiment, the lanes inoculated with the natural biofilm had a relatively higher diversity than the lanes inoculated with *Stigeoclonium*, and a significantly higher biomass production. The algal combinations can better grow in wastewaters because the loss of one alga from the mixture may be compensated by others (Arbib et al., 2014; Renuka et al., 2013), so the mixed culture can have a wide tolerance of external condition changes, such as temperature and irradiance fluctuations under outdoor conditions, or because of complementarity effects (Vanelslander et al., 2009).

However, the use of mixed culture rather than monocultures of benthic algae may pose a problem for discharging biomass-free effluent, the harvest, the resistance of predation and further utilization of the resultant biomass (Guo et al., 2014; Moheimani et al., 2015). First, if an ecosystem contains the planktonic species, it would be hard to separate them from wastewater and discharge biomass-free effluent. Second, the benthic filamentous algae are more resistant to the predation of invertebrate grazers, while the unicellular algae are usually palatable and easily captured by invertebrate grazers (Guo et al., 2014; Wellnitz & Ward, 1998). Third, if the mixed culture contain toxic species such as certain filamentous cyanobacteria, the biomass cannot be used for animal or aquaculture feed (Yan et al., 2012). Fourth, it is more difficult to control the biochemical composition of the biomass in mixed culture than in monoculture. For example, some species in the mixture may produce carbohydrates, while others produce lipids. In this respect it is important to be able to manipulate the ATS biofilms in such a way that preferred species are capable of dominating the biofilm algal community. Therefore, we tried to introduce benthic filamentous algae as dominating species of the outdoor ATS by inoculating them from monoculture (Chapter 4), and investigated the effect of flow rate on algal community composition to optimize the operation process for a high biomass production with a dominance of certain preferred filamentous green algae (Chapter 5).

2.3 Priority effect of benthic algae and biomass production

Generally, the first-arriving species in a community may inhibit or facilitate the establishment of other species that arrive later in the community, causing the colonization order to have a lasting influence on community composition, which is referred to as priority effects (Louette & De Meester, 2007). For an attached cultivation system such as an Algal Turf Scrubber (ATS), a dominance of benthic filamentous algae is usually preferred for cost-efficient harvest and dewatering of the harvested biomass (Craggs et al., 1996; Mulbry et al., 2010). As described in Chapters 4 and 5, the benthic alga *Stigeoclonium* was successfully inoculated to the outdoor ATS and they had a long-lasting priority effect on the algal community for several months, but only at a relatively low flow rate, but there was no significant difference detected in the biomass production and nutrient removal performance between ATS lanes inoculated with one of two *Stigeoclonium* strains, *Pseudanabaena* sp. and the natural biofilm. This was probably masked by the outdoor environmental condition fluctuations, such as temperature and solar irradiance.

However, from the perspective of further utilization of the produced biomass as described above (section 2.2), maintaining the dominance of certain algal species will benefit in producing valuable biomass, such as with high protein or fatty acid content, and disposal of the algal biomass.

2.4 Biomass production, irradiance and temperature in Belgium

As described in Chapter 1, the Algal Turf Scrubbers had a maximal biomass production of 13-61 g DW m^{-2} d^{-1} in other studies (Craggs et al., 1996; Kebede-Westhead et al., 2003; Mulbry et al., 2010), while in the current study it was maximally 4.9 g DW m^{-2} d^{-1} as reported in Chapter 4. Besides the flow rate as elucidated in Chapter 5, temperature and solar irradiance fluctuations were also critical factors in the biomass production (Craggs et al., 1996; Godillot et al., 2001; Kebede-Westhead et al., 2003). It is well known that light intensity and temperature are critical factors in algal photosynthesis (Richmond, 2004). In Chapter 4, the biomass production showed a similar pattern with the fluctuations of weekly average solar irradiance and temperature.

In Belgium, the annual sum of irradiance is only 1000-1100 kWh m^{-2} (KMI: http://www.meteo.be/), with the lowest monthly average of 50 hours of sun in December and the highest of around 200 hours in May to August. Compared to other places including Israel, Australia, China and United States, in which countries the algal mass cultivation has been commercially developed, the numbers of hours and intensity of sunlight in Belgium are much lower (http://solargis.info/doc/free-solar-radiation-maps-GHI). In terms of temperature, although the monthly average temperature varies between 14 and 20 °C from June to September, it is only 1-13 °C from October to May (KMI: http://www.meteo.be/). In Chapter 4, the biomass production of the outdoor ATS decreased sharply from November and the ATS can only start to work from the end of April due to the low temperature and light availability from November to April. Therefore, selecting species with a high light utilization efficiency and tolerance of low temperature will be the logical next step.

2.5 Precipitation and adsorption of phosphorus

In Chapter 4, a sharp decrease in phosphorus concentration was observed whenever horticultural wastewater was refreshed in the outdoor ATS. We suspect that precipitation caused by pH increase was an important mechanism in phosphorus removal from the horticultural wastewater (Craggs et al., 1996). No addition of CO_2 was provided and

thus no pH control was carried out in our outdoor ATS. As a result, the pH increased quickly from 7 to 9 only one day after refreshing the wastewater. In Chapter 5, this was studied further in the indoor experiment by using synthetic wastewater (modified WC medium) as a reference. First, in the synthetic wastewater, EDTA was present to prevent phosphorus precipitation with metal ions (de-Bashan & Bashan, 2004) and the phosphorus concentration decreased more slowly (maximally 1.8 mg PO_4^{3-}-P L^{-1} d^{-1}) than in horticultural wastewater (maximally 5.4 mg PO_4^{3-}-P L^{-1} d^{-1}). Second, the concentrations of Ca^{2+} and Mg^{2+} were 100-160 and 20-40 mg L^{-1} respectively, which enabled the precipitation of phosphate with metal ions. Third, nitrogen uptake rates from horticultural and synthetic wastewater were quite similar (3.7-4.8 mg NO_3^--N L^{-1} d^{-1} and 3.5-5.0 mg NO_3^--N L^{-1} d^{-1} respectively) to each other when the maximal phosphorus removal rate was observed. As indicated in Chapter 3, phosphorus uptake by algal cells highly depends on nitrogen availability and thus nitrogen uptake rate, so for each species the phosphorus uptake rates should be similar to each other in horticultural and synthetic wastewaters. Furthermore, according to the maximal phosphorus uptake rates of *Klebsormidium* and *Stigeoclonium* (Chapter 3), their phosphorus uptake rates during the first day of the indoor experiment (Chapter 5) should be between 0.5 and 1.0 mg PO_4^{3-}-P L^{-1} d^{-1} based on an initial biomass density of 30 mg DW L^{-1}. Therefore, the excess phosphorus removed from horticultural wastewater than from synthetic wastewater must be through other process than assimilation by algal cells.

Moreover, surface adsorption of inorganic phosphorus onto periphyton through the secretion of extracellular polymeric substances (EPS) has been reported as an important mechanism in phosphorus removal by periphyton (Lu et al., 2014; Sheng et al., 2010; Wu et al., 2014). In the study of Lu et al. (2014), EPS contributed to about 46% of phosphorus removal. That is probably because the negatively charged phosphate ions can form strong hydrogen bonds with the polysaccharides, especially at a high pH (e.g. 9) most $H_2PO_4^-$ ions change to HPO_4^{2-} and the hydrogen bonds get stronger than at low pH (Li et al., 2013). Additionally, in the study of Lei (2009) investigating the phosphorus removal mechanisms by *Cladophora glomerata* and *Spirogyra* sp., a precipitation of calcium carbonate caused by pH elevation through photosynthesis of algae was observed first with X-ray Diffraction and then followed by an adsorption of phosphate onto calcium carbonate and the formation of hydroxyapatite. In this study, phosphorus adsorption wasn't investigated, but it must have happened.

For the outdoor ATS in this study, a plastic liner was used as the substrate for the benthic algal community. The plastic material can have positively charged ion ex-

changed sites (Nollet & De Gelder, 2007), while phosphate ions carry negative charges. When the phosphate meets the plastic liner, they can combine together. Therefore, phosphorus could be adsorbed onto the plastic liner and accordingly taken out from wastewater.

Additionally, in Chapter 5 the phosphorus recovered in biomass from horticultural wastewater (46-81%) was much lower than from synthetic wastewater (72-98%) in the indoor experiment and in the outdoor ATS it was higher at high flow rate (86%) than at low flow rate (60%). Similarly, in Chapter 4 the phosphorus recovery rate of the outdoor ATS was 37-60% at a low flow rate. Therefore, the chemical precipitation or adsorption of phosphorus may result in a significant loss of phosphorus from the surface of algal biomass through re-dissolution or desorption caused by pH fluctuations (Gupta & Rastogi, 2008; Lodi et al., 2003; Lu et al., 2014), or during the harvest, such as the centrifugation. Therefore, the chemical precipitation or adsorption of phosphorus should be taken into account when using algae for phosphorus removal and recovery, especially for the cultivation systems without pH control. Accordingly, N/P ratio of wastewater has less influence on phosphorus removal process, especially for the wastewater of a low N/P ratio.

2.6 Feasibility of benthic algae in wastewater treatment in Belgium

The constructed wetland (CW) is a commonly used ecosystem in removing inorganic nutrient and other pollutants from wastewater in Belgium since 1986 (Boets et al., 2011; Rousseau et al., 2004). In the study of Boets et al. (2011), the constructed wetland had an annual average nitrogen removal rate of 520 mg N m^{-2} d^{-1} and phosphorus removal rate of 18 mg P m^{-2} d^{-1} in treating piggery manure. Compared to their results, our ATS had a higher phosphorus removal rate because of the diversified phosphorus removal mechanisms of periphyton, but it had a much lower nitrogen removal rate probably due to the denitrification and volatilization of ammonia in the wetland (Boets et al., 2011; Meers et al., 2008). Furthermore, although the constructed wetland has a higher nitrogen removal rate than our ATS, but the final removal efficiency of nitrogen and phosphorus remained low (31-65% and 26-70% for nitrogen and phosphorus respectively), which made it hard to meet the strict discharge standard (Rousseau et al., 2004). Additionally, the constructed wetland can function in winter although at a lower nutrient removal rate than in other seasons, but for the ATS this is completely infeasible.

Microalgal bacterial floc (MaB-floc) is another newly developed wastewater treating system in removing COD and inorganic nutrient in Belgium (Van Den Hende,

2014; Van den Hende et al., 2014a). The MaB-floc is efficient in reducing COD, BOD from aquaculture, manure and food industrial wastewaters, but low in nitrate and nitrite removal. The biomass produced from MaB-floc can be easily harvested through bioflocculation and dewatered by filtering through 150-250 μm in a cheap way. Moreover, the predation by *Tubifex* sp. caused a significant loss of biomass with 25% of 68 measured biomass productions being negative (Van den Hende et al., 2014a; Van Den Hende et al., 2014b). Additionally, similar to other open systems, all of ATS, CW and MaB-floc face the challenges of evaporation and salinisation (Rousseau et al., 2008; Van Den Hende, 2014).

As shown in this study, the benthic algae are capable of removing nitrogen and phosphorus from the horticultural wastewater to a quite low concentration (less than 1 and 0.1 mg L^{-1} for nitrogen and phosphorus respectively) either under controlled or natural conditions (Chapters 3, 4 and 5). Benthic algae have a high potential capability of resisting to predations because of their large cell/colony size and indigestible cell wall (Guo et al., 2014; Wellnitz & Ward, 1998). In this study, as described in Chapter 4 and 5 (Fig. 6.1), the invertebrate grazers including Chironomid larvae were much less common when filamentous green algae dominated on the ATS, while being abundant when the unicellular species such as *Desmodesmus* and *Scenedesmus* were the dominants.

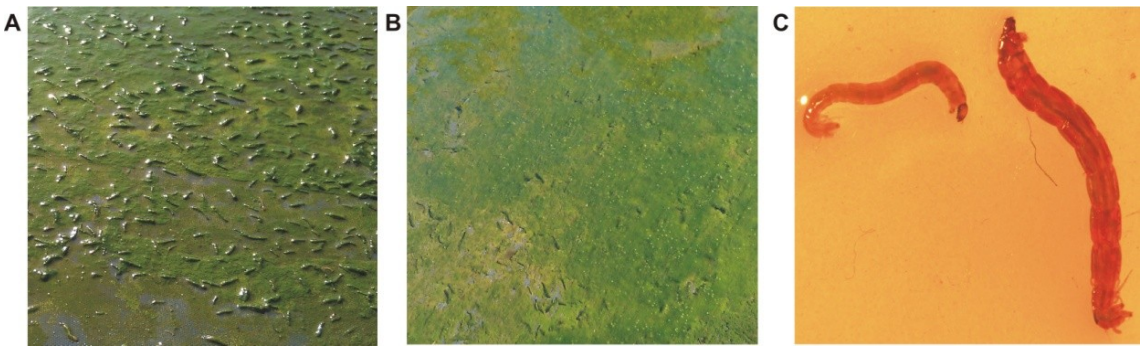

Fig. 6.1 A: the ATS lane dominated by unicellular algae; B: the ATS lane dominated by filamentous algae; C: Chironomid larvae found inside the green rods of A.

In this study, the stress was put on the inorganic nitrogen and phosphorus removal horticultural wastewater, and the benthic algae were proved to be capable of reducing nitrogen and phosphorus to low levels. Thus, the benthic algae and their community can be applied in removing inorganic nitrogen and phosphorus from wastewaters rich of inorganic nitrogen and phosphorus, such as agricultural, horticultural, manure, aquaculture wastewaters and the second effluent of wastewater treatment plants. Moreover, other pollutants, such as heavy metals, can also be efficiently re-

moved by benthic filamentous algae and their communities (Axtell et al., 2003; Ji et al., 2012; Pawlik-Skowrońska, 2001).

3. Future research perspectives and challenges

3.1 Opportunities and limitations of benthic algae in wastewater treatment

Algae have the capacity of growing fast and efficiently assimilating nitrogen and phosphorus from wastewater with low operational costs and no secondary pollutions (Abdel-Raouf et al., 2012; Aslan & Kapdan, 2006). In recent decades, biological wastewater treatment with microalgae is gaining worldwide acceptances. However, the harvesting and dewatering of microalgae from wastewater are energy and time consuming and remain the main bottle-neck for the large-scale application. Additionally, the resistance of microalgae to invertebrate grazers is another widespread problem in mass cultivation.

Benthic filamentous algae have the advantage of growing attached to substrates using a holdfast or producing mucilage (Mulbry & Wilkie, 2001; Scott et al., 1996; Sekar et al., 2004). Therefore, attached cultivation systems have been developed to solve the high energy consumption problem of harvesting (Kesaano & Sims, 2014). Furthermore, benthic filamentous algae with large cell/colony size and thick cell wall of high cellulose content have been reported to be more resistant to grazers than unicellular species (Guo et al., 2014; Wang et al., 2014; Wang et al., 2013b; Wellnitz & Ward, 1998), and become a promising option for large-scale applications. Based on these characteristics, in recently years, benthic filamentous algae are getting attractive in wastewater treatment and large-scale cultivation (Guo et al., 2014; Markou & Georgakakis, 2011; Roberts et al., 2013; Wang et al., 2013a). Accordingly, several kinds of simple-constructed attached cultivation systems have been developed and widely used in wastewater treatment (Kesaano & Sims, 2014).

Due to their relatively low growth rate compared to unicellular algae (Griffiths et al., 2012; Liu & Vyverman, 2015), it is hard for benthic filamentous algae to establish themselves because of competition with faster-growing unicellular microalgae for nutrient uptake in the mixture and easily lose their dominance to the unicellular species as described in Chapter 4, but the priority effect can guarantee the dominance to an extent. Furthermore, in an attached cultivation system such as Algal Turf Scrubber used in this

study, the attachment of benthic filamentous algae can be easily broken by external factors, for instance the shear stress produced from water current or heavy rain. Therefore, how to maintain the dominance of benthic filamentous algae and maximize their biomass production of the periphyton community will be main challenges for their further large-scale application in the future. Some considerations on this aspect are discussed below.

3.2 Application considerations of benthic filamentous algae based cultivation systems

Biofilm-based systems are widespread applications of benthic filamentous algae and the best ways to make full use of the advantages of benthic filamentous algae either in mass cultivation or wastewater treatment (Kesaano & Sims, 2014). Except for the factors including temperature and solar irradiance mentioned above, there are other important factors for an efficient and success cultivation system of benthic filamentous algae.

Benthic filamentous algae grow in biofilm via cell adhesion to a surface, so the attachment strength is vital in the biofilm formation and continuous growth. In addition to the substrate material and texture (Gross et al., 2015; Johnson & Wen, 2010), the operation parameters are also critical. For instance, the shear stress caused by current can easily break the biofilm attachment to the substrate and heavy rain can cause fatal damage to the biofilm under outdoor conditions, accordingly it will reduce the biomass production and nutrient removal efficiency. Therefore, minimizing the breakage of biofilm from shear stress, such as using an appropriate flow rate setting, substrate materials with positive charges and the algal community with a high EPS secretion, should be taken into consideration.

Except for the external factors of breaking the biofilm attachment, its natural sloughing happens as well following the biomass accumulation (Gross et al., 2015; Sandefur et al., 2011). Therefore, a regular harvest is necessary to remove the excessive biomass and stimulate the further biofilm growth. Accordingly, a proper harvesting frequency is critical in maximizing the biomass production. Based on the biofilm growth cycles in different seasons, a short harvesting interval is usually preferred in warm seasons, while a longer harvesting interval and/or larger inoculum will benefit biomass production in cold seasons.

For any open cultivation system and biofilm community, the interaction and competition between the target algal species and other microorganisms are inevitable (Di Pippo et al., 2014). Although the primary objective of wastewater treatment is to remove nutrient and other pollutants, the resultant biomass with nontoxic and valuable composition is always preferable. Therefore, it is recommendable to maintain certain benthic filamentous algae as the dominating species in the community for the integration of wastewater treatment and valuable biomass production.

4. Overall conclusions

This work presents the biochemical composition of several benthic filamentous algae under different culture conditions and an attached system for horticultural wastewater treatment and nutrient recovery. The main contributions of this research can be summarized as: (i) having provided an insight in species-specific protein content and fatty acid profile changes of four benthic filamentous green algae during growth phases and under nitrogen deprivation or dark exposure; (ii) having explored the effects of N/P ratio of growth medium on algal growth, nutrient removal and nutrient composition of four benthic algae; (iii) having investigated the algal community diversity and biomass production of an outdoor ATS in different seasons in Belgium; (iv) having exploited the priority effects of several benthic filamentous algae on algal community and the consequent biomass production and nutrient removal on ATS; (v) having investigated the effects of flow rate on algal community, biomass production and nutrient removal. The comparison of different algal species in growth, biochemical composition and nutrient removal capacity can benefit in selecting the robust species for large-scale application. The integration of nutrient removal and biomass production of benthic filamentous algae based attached cultivation system provides several opportunities (e.g. resistance to predation, ease in harvest and production of valuable biomass) for further research and applications. Further optimization of the benthic filamentous algae based bioreactor regarding nutrient removal and recovery, biomass production and further utilization are needed to set the stage for their large and/or commercial scale applications.

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Summary

Samenvatting

Summary

In recent years, microalgae have received increasing attention in biotechnology research with regards to producing protein and polyunsaturated fatty acids (PUFAs) as human and animal nutrition and removing nutrient from wastewater. Because of their large cell/colony size and thick cell wall with high cellulose content, benthic filamentous algae and their communities have potentials of being more resistant to invertebrate grazers and easier and cheaper to harvest than the unicellular algae. Accordingly, benthic algae based bioreactors have been developed and are widely accepted in wastewater treatment.

Algal biomass production, biochemical composition and nutrient removal capacity from wastewater are species-specific and highly depend on the wastewater composition (N/P type, concentration and ratio) and operation conditions (light, temperature, harvesting frequency, etc.). For an attached cultivation system, shear stress induced by water current is also crucial in biofilm formation on the substrate. Therefore, selection of the appropriate algal species and optimization of operational conditions will be vital in maintaining the dominance of benthic algae and improving biomass production and nutrient removal efficiency in a time and cost efficient way. Accordingly, four research chapters explore the performances of several benthic filamentous algae and their communities in nutrient removal from horticultural wastewater and the consequent biomass production and biochemical composition through indoor flasks and outdoor Algal Turf Scrubber (ATS).

In Chapter 1, the background information of algae-based technologies in nutrient removal from wastewater and relevant factors of the cultivation process was presented.

In Chapter 2, biomass of four benthic filamentous green algae *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. was harvested from exponential growth phase, stationary phase, nitrogen deprivation and dark treatment. The four species had significantly different total protein content and fatty acid profiles. They had a protein content of 29-49% of dry weight in the exponential phase. The increasing culture age, nitrogen deprivation and dark treatment significantly increased their polyunsaturated fatty acids (PUFAs) percentage of total fatty acids (TFAs) from 21-58% to 55-87% of these four species. *Klebsormidium* spp. and *Stigeoclonium* sp. LJ1 can be good potential sources of C18:2ω6 and C18:3ω3 respectively.

In Chapter 3, three benthic filamentous green algae *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. and one cyanobacterium *Pseudanabaena* sp. were cultivated under varying N/P conditions in batch model. The four species could adapt to a wide range of N/P ratio and the proper N/P ratios for nitrogen and phosphorus removal were 7 to 10, 5 to 12, 5 to 15 and 7 to 20 for *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 and *Pseudanabaena* sp. respectively. The N/P ratio significantly influenced the algal growth and phosphorus uptake process, while nitrogen uptake process was less influenced by N/P ratio. The nitrogen and phosphorus content varied greatly following the increase of N/P ratio from 1:1 to 20:1 and the nitrogen and phosphorus recovery rates were 73-91% and 78-99% respectively. In addition, *Stigeoclonium* sp. LJ2 had a high capability of removing phosphorus from wastewaters of low N/P ratio, while *Pseudanabaena* sp. was highly suitable for removing nitrogen from wastewaters with high N/P ratio and high nitrogen concentration. A better understanding of the physiological diversity and stoichiometry of benthic algae and their habitat requirements can contribute significantly to selecting the appropriate algal strains or combinations for the large-scale application in nutrient removal from wastewater.

In Chapter 4, 1 m^2 scale ATS was built to investigate the algal community, biomass production and nutrient removal performance of biofilms with different inoculums of benthic algal communities following the seasonal variations in Belgium. The biomass production in this study was relatively low, which was 0.1-1.9 g dry weight m^{-2} d^{-1} in spring, 0.7-4.9 g dry weight m^{-2} d^{-1} in summer and 0.2-1.6 g dry weight m^{-2} d^{-1} in autumn. Ash, carbon, nitrogen and phosphorus content was about 13-27%, 41-49%, 6-9% and 1.3-2.3% of dry weight respectively. At a low flow rate of 2 L min^{-1}, the benthic filamentous algae *Stigeoclonium* had a longer-lasting dominance on the ATS than at high flow rate. In addition, it indicated that temperature and solar irradiance were the main limiting factors of biomass production and nutrient removal under the natural conditions of Belgium and flow rate was a potential factor influencing algal community, biomass production and nutrient removal of the ATS.

In Chapter 5, to assess the potential of the benthic filamentous algae in nutrient removal and reclaim from horticultural wastewater, three species *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. were cultivated in 250 ml laboratory flasks in monoculture and mixture as well as in 1 m^2 scale outdoor ATS with different flow rates. *Stigeoclonium* spp. Competed well with the naturally occurring wastewater microalgae and contributed to most of the biomass production both in laboratory flasks and outdoor ATS with a relatively low flow rate of 2-6 L min^{-1} (3-9 cm s^{-1}). The low flow rate facili-

tated the dominance of benthic filamentous algae, while the high flow rate enhanced the biomass production and nitrogen removal on the ATS. Additionally, phosphorus removal was less influenced by flow rate because of chemical precipitation and/or surface adsorption onto the periphyton or the plastic liner.

Chapter 6 finishes with a general discussion of this work and proposes further opportunities and challenges of benthic algal community in large scale applications. To conclude, the main strengths of the presented benthic algal community are the priority effects of certain species on the benthic algal community composition and the differences in biomass production and nutrient removal at various flow rates. Further optimizations regarding selecting the species with high light utilization efficiency and tolerance of low temperature and the appropriate substrates are needed to set the stage for the commercial applications.

Samenvatting

In de laatste jaren is er verhoogde aandacht vanuit de biotechnologie voor microalgen met het oog op de productie van proteïnen en poly-onverzadigde vetzuren (PUFAs) voor menselijke en dierlijke voeding, en het verwijderen van nutriënten uit afvalwater. Door het grote formaat van hun cellen/kolonies en hun dikke celwand met hoog cellulose gehalte, hebben benthische filamenteuze algen en hun gemeenschappen een potentieel voor hogere resistentie tegen grazers en eenvoudigere en goedkopere oogstmethodes dan eencellige algen. Aldus werden bioreactoren gebaseerd op benthische algen ontwikkeld en krijgen deze meer en meer aandacht in afvalwater zuivering

Biomassaproductie, biochemische samenstelling, en capaciteit voor het verwijderen van nutriënten uit afvalwater zijn soortspecifiek en hangen sterk af van de samenstelling van het afvalwater (soort, concentratie en ratio van N en P) en de operationele condities (licht, temperatuur, frequentie van oogst, etc.). Voor cultivatiesystemen met vastgehechte algen speelt ook schuifspanning, geïnduceerd door het stromen van het water, een cruciale rol in de vorming van biofilms op het substraat. Daarom is selectie van de geschikte soorten algen en optimalisatie van operationele condities van vitaal belang in het behouden van de dominantie van benthische algen en het verhogen van biomassa productie en verwijdering van nutriënten op een efficiënte manier. In zes hoofdstukken wordt onderzoek gedaan naar de prestaties van verschillende benthische filamenteuze algen en hun gemeenschappen in het verwijderen van nutriënten uit afvalwater afkomstig van tuinbouwactiviteiten, en de productie en biochemische samenstelling van biomassa die daaruit volgt, zowel in laboflessen, als in een openlucht Algal Turf Scrubber (ATS).

In Hoofdstuk 1 wordt de achtergrondinformatie van technologieën gebaseerd op algen voor het verwijderen van nutriënten uit afvalwater, en de relevante factoren van het cultivatie proces geschetst.

In Hoofdstuk 2 werd biomassa geoogst van de vier benthische filamenteuze groenwieren *Klebsormidium* sp. LJ1, *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1 and *Uronema* sp. in exponentiële en stationaire groeifase, in stikstof deprivatie, en in donker behandeling. De vier soorten verschilden significant in hun proteïne- en vetzuursamenstelling. Ze hadden een proteïnesamenstelling van 29-49% van het drooggewicht in exponentiële fase. Verlengde cultivatie, stikstof deprivatie en donker

behandeling verhoogden allen het aandeel van poly-onverzadigde vetzuren (PUFAs) in de totale vetzuursamenstelling (TFAs) van 24-58% tot 55-87% in alle vier de soorten. *Klebsormidium* spp. en *Stigeoclonium* sp. LJ1 hebben potentieel als bron van C18:2ω6 en C18:3ω3 respectievelijk.

In Hoofdstuk 3 werden drie filamenteuze groenwieren *Klebsormidium* sp. LJ2 and *Stigeoclonium* spp. en een cyanobacteria *Pseudanabaena* sp. gecultiveerd in variërende N/P condities in batch model. De vier soorten konden zich aanpassen aan een breed scala aan N/P ratios, en de beste N/P ratios voor het verwijderen van stikstof en fosfor waren 7 tot 10, 5 tot 12, 5 tot 15 and 7 tot 20 voor *Klebsormidium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Stigeoclonium* sp. LJ2 en *Pseudanabaena* sp. respectievelijk. De N/P ratio had een significante invloed op de groei van de algen en hun opname van fosfor, terwijl stikstofopname minder beïnvloed werd door de N/P ratio. Stikstof en fosfor gehalte varieerden zeer sterk, en volgden de stijging in N/P ratio van 1:1 tot 20:1, en de herwinningefficiëntie voor stikstof en fosfor was 73-91% en 78-99% respectievelijk. Bovendien had *Stigeoclonium* sp. LJ2 een hoge capaciteit om fosfor te verwijderen uit afvalwater met lage N/P ratio, terwijl *Pseudanabaena* sp. zeer geschikt was voor het verwijderen van stikstof uit afvalwater met hoge N/P ratio. Een beter begrip van de fysiologische diversiteit en stoichiometrie van benthische algen en hun habitatvereisten kan significant bijdragen aan het selecteren van de geschikte soorten of combinaties van algen voor grootschalige toepassing in het verwijderen van nutriënten uit afvalwater.

In Hoofdstuk 4 werd een 1 m^2 ATS geconstrueerd om onderzoek te doen naar de algen gemeenschappen, biomassa productie en verwijdering van nutriënten door biofilms met verschillende inocula van benthische algen gemeenschappen gedurende de seizoenale variaties in België. De biomassa productie in deze studie was relatief laag, met 0.1-1.9 g drooggewicht m^{-2} d^{-1} in de voorjaar, 0.7-4.9 g drooggewicht m^{-2} d^{-1} in de zomer en 0.2-1.6 g drooggewicht m^{-2} d^{-1} in de herfst. As-, koolstof-, stikstof- en fosforgehalte bedroegen 13-27%, 41-49%, 6-9% en 1.3-2.3% van het drooggewicht respectievelijk. Bij een laag debiet van 2 L min^{-1} domineerden de filamenteuze algen *Stigeoclonium* langer op de ATS dan bij een hoger debiet. Bovendien leek het erop dat de temperatuur de belangrijkste limiterende factor was voor biomassa productie en verwijdering van nutriënten onder natuurlijk omstandigheden in België. Debiet was een andere potentiële factor die de soortsamenstelling, biomassa productie en nutriënt verwijdering van de ATS kon beïnvloeden.

In Hoofdstuk 5 werden drie soorten (*Klebsormidium* sp. LJ2 en *Stigeoclonium* spp.) gecultiveerd van 250 ml laboflessen in monocultuur en mengeling, tot 1 m² openlucht ATS met verschillende stroomsnelheden, om het potentieel van benthische filamenteuze algen voor het verwijderen en herwinnen van nutriënten uit afvalwater afkomstig van sierteelt activiteiten to onderzoeken. *Stigeoclonium* spp. konden goed concurreren met microalgen afkomstig uit het afvalwater en droegen het meest bij aan de biomassa productie in zowel de laboflessen als de openlucht ATS met relatief laag debiet van 2-6 L min^{-1} (3-9 cm s^{-1}). Het lage debiet faciliteerde de dominantie van filamenteuze algen, terwijl een hoog debiet de biomassa productie en stikstof verwijdering in de ATS verhoogde. Verwijdering van fosfor werd minder beïnvloed door het debiet, waarschijnlijk door chemische precipitatie en/of adsorptie op het oppervlakte van het periphyton of de plastic voering.

Hoofdstuk 6 eindigt met een algemene discussie over dit werk en bespreekt de mogelijkheden en uitdagingen bij het gebruik van benthische algen in grootschalige applicaties. In conclusie, de voornaamste voordelen van de voorgestelde fytobenthische gemeenschap zijn: prioriteitseffecten van bepaalde soorten op de samenstelling van de fytobenthische gemeenschap en verschillen in biomassa productie en snelheid waarmee nutriënt worden verwijderd bij verschillende stroomsnelheden. Een verdere optimalisatie inzake een selectie van soorten aangepast aan lagere lichtcondities en temperaturen en geschikte substraten, is noodzakelijk om commercialisatie mogelijk te maken.

Appendix

Raw data of the outdoor experiment on Algal Turf Scrubber (ATS)

Table A1 The biomass density (g DW m^{-2}) on different days of the ATS lanes inoculated with natural biofilm in different seasons of 2013 (spring: May to June; summer: July to August; autumn: September to October; winter: November to December). 1, 2 and 3 stand for the triplicates. A, B represent the upper and lower portions of the lane, respectively. This was presented in Fig. 4.4 in Chapter 4.

Season	Day	1		2		3	
		A	B	A	B	A	B
Spring	0	2.5	5.3	1.0	7.6	0.1	0.5
	2	3.2	6.5	2.1	9.2	0.7	1.0
	4	4.4	6.3	6.4	17.7	2.1	1.6
	6	7.5	5.9	12.6	24.3	2.8	2.3
	8	9.5	7.8	18.2	27.4	7.4	5.3
	10	11.6	8.7	23.2	32.8	10.1	7.5
	12	12.9	7.0	23.3	28.1	12.3	8.9
	14	15.5	9.6	42.0	27.0	12.8	8.9
	16	17.3	9.3	33.6	10.2	17.6	9.3
Summer	0	1.9	1.4	5.3	5.4	3.5	7.1
	3	5.5	2.5	8.8	8.8	11.4	11.4
	6	9.4	4.1	21.1	14.6	25.4	17.3
	9	17.7	11.4	31.4	26.9	39.4	25.7
	12	28.9	16.5	38.9	41.0	53.8	30.8
	15	33.7	12.4	48.5	38.9	60.9	30.1
	18	51.0	17.0	51.7	44.7	71.9	33.1
	21	48.0	19.3	60.3	24.0	48.6	35.3
Autumn	0	4.4	3.0	5.4	6.0	10.1	5.2
	3	11.7	5.3	10.5	8.6	17.1	14.3
	6	6.3	8.6	20.0	19.6	24.0	14.5
	9	9.4	12.2	30.6	20.1	24.7	15.1
	12	8.7	8.6	34.8	19.5	24.9	22.2
	15	6.9	7.9	31.0	16.9	15.9	17.4
Winter	0	0.3	2.2	0.8	1.8	0.7	1.6
	3	1.9	2.4	0.7	1.9	1.1	1.7
	6	1.7	4.1	0.6	3.3	1.4	2.3
	9	1.0	4.4	0.7	3.0	1.8	2.3
	12	2.1	5.7	1.4	4.5	2.8	2.2

15	1.5	1.5	2.7	6.4	2.6	2.5
18	3.3	5.6	4.6	8.0	4.7	5.2

Table A2 The biomass production (g DW m^{-2} d^{-1}) of the ATS inoculated with natural biofilm in different months of 2012, 2013 and 2014. 1, 2 and 3 represent triplicates. This was presented in Fig. 4.5 in Chapter 4.

Date	Biomass production (g m^{-2} d^{-1})		
	1	2	3
14/08/2012	3.1	1.2	0.6
22/08/2012	1.0	1.1	1.2
2/09/2012	0.5	0.5	1.1
11/09/2012	0.5	1.7	1.1
18/09/2012	0.7	1.0	3.1
25/09/2012	0.9	1.2	0.8
15/10/2012	0.1	1.4	0.3
22/10/2012	0.4	1.1	0.6
29/10/2012	0.5	0.8	0.5
6/11/2012	0.4	0.5	0.2
13/11/2012	0.5	0.8	0.1
20/11/2012	0.6	0.6	0.1
27/11/2012	0.5	0.3	0.1
22/05/2013	0.1	0.1	0.0
29/05/2013	0.1	0.1	0.1
5/06/2013	0.3	0.4	0.3
12/06/2013	1.8	0.8	1.1
19/06/2013	0.7	0.9	1.0
26/06/2013	0.3	0.4	0.5
3/07/2013	0.1	1.2	1.3
10/07/2013	1.9	1.7	2.7
17/07/2013	0.5	1.5	1.8
24/07/2013	2.0	2.5	1.5
31/07/2013	0.9	0.4	0.7
7/08/2013	1.9	2.8	2.4
14/08/2013	1.3	2.4	1.5
21/08/2013	1.7	2.0	1.4
28/08/2013	1.1	1.3	1.4
11/09/2013	1.7	1.0	1.8
25/09/2013	1.0	0.9	1.6
2/10/2013	1.9	2.4	2.0
9/10/2013	1.0	1.5	1.4
23/10/2013	0.4	0.3	0.3
6/11/2013	0.3	0.4	0.1
20/11/2013	0.0	0.2	0.0

4/12/2013	0.0	0.2	0.0
14/05/2014	2.4	0.8	2.6
21/05/2014	5.7	2.6	2.8
27/05/2014	4.9	5.3	4.4
4/06/2014	0.8	1.6	1.4
11/06/2014	2.2	2.5	3.1
18/06/2014	4.1	4.0	1.8
25/06/2014	3.8	2.3	3.5
2/07/2014	1.6	2.8	2.3
10/07/2014	1.2	0.6	1.7
16/07/2014	3.5	1.5	2.4
23/07/2014	4.6	1.3	2.0
30/07/2014	1.8	2.0	1.6
6/08/2014	5.0	2.2	3.1
13/08/2014	1.5	1.7	1.7
20/08/2014	2.0	0.9	1.7
27/08/2014	1.4	1.1	0.9
3/09/2014	4.0	4.2	3.3
10/09/2014	2.4	2.4	2.6
17/09/2014	1.6	1.8	1.2
24/09/2014	1.6	1.2	1.5
1/10/2014	0.9	1.2	0.6
8/10/2014	0.4	0.6	0.4
15/10/2014	0.9	1.1	0.6
22/10/2014	0.5	0.4	0.6
30/10/2014	0.5	0.7	0.9
5/11/2014	0.4	0.4	0.3
12/11/2014	1.2	0.8	1.0
19/11/2014	0.7	0.3	0.7
26/11/2014	0.7	0.5	0.4
3/12/2014	0.8	0.6	0.4

Table A3 The biomass production (g DW m^{-2} d^{-1}) of the ATS with different inoculums in 2014 (A-D: *Stigeoclonium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Pseudanabaena* sp. and natural biofilm). This was presented in Fig. 4.6 in Chapter 4.

Date	Inoculum	Biomass production (g m^{-2} d^{-1})		
		1	2	3
14/05/2014	D	2.4	0.8	2.6
21/05/2014	D	5.7	2.6	2.8
27/05/2014	D	4.9	5.3	4.4
4/06/2014	D	0.8	1.6	1.4
11/06/2014	D	2.2	2.5	3.1
18/06/2014	D	4.1	4.0	1.8

25/06/2014	D	3.8	2.3	3.5
2/07/2014	D	1.6	2.8	2.3
10/07/2014	D	1.2	0.6	1.7
16/07/2014	D	3.5	1.5	2.4
23/07/2014	A	0.6	1.0	1.6
	B	2.1	1.6	1.7
	C	1.7	2.3	1.3
	D	4.6	1.3	2.0
30/07/2014	A	0.6	0.2	1.4
	B	1.6	2.0	2.3
	C	1.6	1.1	1.4
	D	1.8	2.0	1.6
6/08/2014	A	1.4	2.6	2.2
	B	2.3	2.6	2.6
	C	1.9	1.9	3.0
	D	5.0	2.2	3.1
13/08/2014	A	0.8	0.4	0.8
	B	1.7	1.3	2.1
	C	1.1	0.8	1.3
	D	1.5	1.7	1.7
20/08/2014	D	2.0	0.9	1.7
27/08/2014	D	1.4	1.1	0.9
3/09/2014	D	4.0	4.2	3.3
10/09/2014	D	2.4	2.4	2.6
17/09/2014	D	1.6	1.8	1.2
24/09/2014	A	1.1	0.4	0.6
	B	0.5	0.9	0.8
	C	1.1	0.4	0.4
	D	1.6	1.2	1.5
1/10/2014	A	0.6	0.7	0.7
	B	0.7	0.6	0.5
	C	0.6	0.8	0.6
	D	0.9	1.2	0.6
8/10/2014	A	0.8	1.5	0.9
	B	0.6	1.8	0.4
	C	0.8	0.9	0.3
	D	0.4	0.6	0.4
15/10/2014	A	1.3	1.3	0.8
	B	0.8	0.9	0.9
	C	0.8	1.1	0.5
	D	0.9	1.1	0.6
22/10/2014	A	0.7	1.2	0.6
	B	0.8	0.6	0.6
	C	0.4	0.6	0.4
	D	0.5	0.4	0.6
30/10/2014	A	1.0	0.7	0.4

	B	0.7	0.6	1.0
	C	0.8	0.7	0.4
	D	0.5	0.7	0.9
5/11/2014	A	1.0	0.7	0.8
	B	1.0	0.6	1.1
	C	0.9	0.9	0.5
	D	0.4	0.4	0.3
12/11/2014	A	1.1	1.3	0.9
	B	0.6	1.4	1.2
	C	0.9	1.1	1.1
	D	1.2	0.8	1.0
19/11/2014	A	0.5	0.8	0.7
	B	0.1	0.8	0.9
	C	0.7	0.7	0.2
	D	0.7	0.3	0.7
26/11/2014	A	1.1	0.8	0.6
	B	0.2	0.5	0.4
	C	0.5	1.0	0.7
	D	0.7	0.5	0.4
3/12/2014	A	0.2	0.7	0.8
	B	0.1	0.4	0.4
	C	0.3	0.8	0.2
	D	0.8	0.6	0.4

Table A4 Nitrogen and phosphorus removal efficiency (%) of the ATS lanes with different inoculums over time in summer and autumn of 2014 (A-D: *Stigeoclonium* sp. LJ2, *Stigeoclonium* sp. LJ1, *Pseudanabaena* sp. and natural biofilm). There was small but continuous leakage in one lane inoculated with *Stigeoclonium* sp. LJ2 and one inoculated with natural biofilm, so only two replicates were presented. This was presented in Fig. 4.7 in Chapter 4.

	Day	A1	A2	B1	B2	B3	C1	C2	C3	D1	D2
N removal in summer	29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	31	56.1	48.7	40.2	63.8	51.2	64.1	55.2	59.2	66.6	81.2
	34	97.2	94.7	97.1	95.5	94.8	97.3	99.7	97.2	97.8	98.1
	38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	40	18.6	4.1	16.5	51.4	32.1	13.6	16.7	14.9	31.5	45.4
	42	52.7	34.2	36.7	81.4	49.0	46.0	48.1	48.2	55.7	71.9
	44	76.4	50.1	46.8	94.8	67.1	66.1	64.2	65.9	76.6	88.4
P removal in summer	29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	31	82.4	69.8	87.3	58.7	62.1	55.0	79.9	68.0	80.6	51.4
	34	83.3	44.7	82.6	44.6	73.3	90.5	70.1	81.2	88.7	87.7
	38	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	40	30.1	41.7	50.9	47.9	49.2	60.6	37.1	46.7	60.6	56.0

	42	45.8	48.3	54.1	61.3	58.1	75.4	62.0	65.1	65.7	62.9
	44	53.8	61.9	63.6	66.7	63.9	68.9	71.8	72.3	78.5	81.0
N removal in autumn	14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	16	0.0	17.4	6.4	9.7	7.9	3.1	8.4	6.2	3.0	3.7
	18	21.7	43.7	12.3	26.7	27.0	24.7	25.4	25.7	34.5	32.7
	20	32.4	57.5	18.9	31.3	33.8	23.7	31.6	28.1	38.3	45.0
	22	39.6	68.4	23.7	36.3	38.9	31.4	37.9	35.6	38.5	54.0
	24	53.1	82.9	32.9	38.9	44.3	37.0	41.1	40.2	67.3	68.0
	26	65.0	90.9	38.0	48.7	54.1	45.0	46.9	47.0	72.0	78.0
	28	78.3	98.4	46.3	58.4	64.4	58.4	54.5	58.4	79.5	88.4
	31	98.6	100.0	59.2	79.1	77.6	73.3	54.6	64.0	88.9	99.3
	33	94.5	100.0	71.1	92.0	88.0	84.5	62.1	74.1	94.7	97.2
P removal in autumn	14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	16	38.1	36.1	23.6	57.4	84.7	31.2	52.6	42.2	89.2	77.1
	18	63.3	79.0	66.7	75.8	89.1	68.0	68.4	68.9	80.6	81.1
	20	60.9	72.8	41.8	67.8	91.9	61.9	71.0	66.6	87.0	86.8
	22	61.9	74.2	21.9	69.0	94.5	56.5	74.5	65.6	81.0	88.1
	24	81.8	88.7	69.7	80.6	93.0	84.6	79.3	82.9	90.4	85.3
	26	83.4	84.0	67.7	85.6	94.0	82.6	78.4	81.7	89.2	83.7
	28	83.4	87.1	88.4	87.9	94.9	75.8	80.1	80.0	88.5	85.2
	31	87.6	84.7	94.5	91.8	94.9	87.3	85.8	86.6	88.8	86.2
	33	83.9	80.1	95.1	93.9	90.6	93.2	93.7	93.7	91.3	92.0

Table A5 Biomass production (g DW m^{-2} d^{-1}) of the ATS with inoculums of natural biofilms (A) and *Stigeoclonium* sp. (B) and the weekly average temperature (T, °C) and solar irradiance (RAD, kWh m^{-2}) in 2013. This was presented in Fig. 4.8 in Chapter 4.

Date	A1	A2	A3	B1	B2	B3	T (°C)	RAD (kWh m^{-2})
22/05/2013	0.1	0.1	0.0	0.0	0.1	0.1	10.2	2.4
29/05/2013	0.1	0.1	0.1	0.0	0.1	0.1	10.1	4.9
5/06/2013	0.3	0.4	0.3	0.5	0.8	0.7	12.4	5.1
12/06/2013	1.8	0.8	1.1	1.1	0.8	1.0	16.2	6.2
19/06/2013	0.7	0.9	1.0	1.0	0.8	1.3	17.7	4.9
26/06/2013	0.3	0.4	0.5	0.8	0.4	1.2	15.4	3.8
3/07/2013	0.1	1.2	1.3	0.3	1.1	1.0	15.4	4.0
10/07/2013	1.9	1.7	2.7	1.1	1.1	1.0	18.9	6.2
17/07/2013	0.5	1.5	1.8	0.5	1.0	1.0	17.9	5.9
24/07/2013	2.0	2.5	1.5	1.4	2.2	1.7	22.1	6.3
31/07/2013	0.9	0.4	0.7	0.5	0.6	0.8	20.3	4.5
7/08/2013	1.9	2.8	2.4	2.1	1.9	1.5	20.9	5.1
14/08/2013	1.3	2.4	1.5	1.7	2.3	1.5	16.3	4.7
21/08/2013	1.7	2.0	1.4	1.3	1.2	0.9	18.1	4.4
28/08/2013	1.1	1.3	1.4	1.5	1.1	0.7	18.0	3.7
11/09/2013	1.7	1.0	1.8	1.4	1.0	0.7	16.5	3.8
25/09/2013	1.0	0.9	1.6	0.9	0.6	0.5	13.8	2.5

2/10/2013	1.9	2.4	2.0	2.1	1.7	1.5	13.0	3.5
9/10/2013	1.0	1.5	1.4	0.9	0.9	1.7	14.2	2.4
23/10/2013	0.4	0.3	0.3	0.2	0.5	0.5	12.1	1.4
6/11/2013	0.3	0.4	0.1	0.1	0.2	0.1	11.6	1.3
20/11/2013	0.0	0.2	0.0	0.0	0.0	0.0	6.5	0.9
4/12/2013	0.0	0.2	0.0	0.0	0.1	0.0	5.3	0.7

Table A6 Biomass production (g DW m^{-2} d^{-1}) of the ATS lanes with inoculum of *Klebsormidium* sp. and *Stigeoclonium* spp. at flow rate of 2, 4, 6 and 8 L min^{-1} in April-June, 2015. This was presented in Fig. 5.5 in Chapter 5.

Week	Flow rate (L min^{-1})	Biomass production (g DW m^{-2} d^{-1})		
		1	2	3
1	2	0.8	0.7	1.1
	4	0.8	0.5	0.9
	6	1.3	1.1	1.0
	8	1.3	0.9	1.3
2	2	1.5	1.5	1.3
	4	1.5	1.4	1.9
	6	1.3	1.3	1.5
	8	2.3	1.1	2.3
3	2	1.0	1.0	1.3
	4	1.4	1.3	1.1
	6	1.7	1.4	1.3
	8	2.5	1.8	1.5
4	2	1.3	1.6	2.3
	4	2.2	1.8	1.8
	6	1.7	1.8	2.1
	8	1.5	1.5	2.7
5	2	1.5	1.3	1.4
	4	2.1	2.0	1.7
	6	2.6	1.6	2.5
	8	2.1	1.9	2.0

Table A7 NO_3^--N and PO_4^{3-}-P concentration (mg L^{-1}) changes (in triplicate) of the outdoor ATS at flow rates of 2-8 L min^{-1} over time. This was presented in Fig. 5.7 of Chapter 5.

Date	Flow rate (L min^{-1})	NO_3^--N (mg L^{-1})			PO_4^{3-}-P (mg L^{-1})		
		1	2	3	1	2	3
18/05/2015	2	32.4	34.1	34.2	9.5	9.5	9.5
	4	31.6	32.9	33.6	9.5	9.5	9.5
	6	31.9	34.9	32.0	9.5	9.5	9.5
	8	31.7	31.9	32.0	9.5	9.5	9.5

20/05/2015	2	26.6	29.9	30.0	1.8	3.3	2.1
	4	27.1	30.4	25.6	2.7	5.4	3.1
	6	27.8	32.4	26.7	3.2	3.4	2.1
	8	26.1	26.9	27.7	1.3	2.5	1.2
22/05/2015	2	23.3	30.1	27.0	1.3	3.0	2.6
	4	26.2	30.8	23.8	2.2	4.1	2.8
	6	25.4	31.2	27.1	2.6	3.1	3.1
	8	21.9	25.1	25.5	1.1	2.9	1.9
25/05/2015	2	11.6	22.4	26.0	1.5	3.5	4.6
	4	21.0	30.1	16.9	4.2	3.5	3.7
	6	17.2	26.0	21.3	3.6	3.1	4.7
	8	12.2	15.0	16.1	1.0	3.0	2.5
27/05/2015	2	7.5	19.1	21.7	1.2	1.2	1.4
	4	16.6	20.7	15.4	1.8	1.0	1.7
	6	13.6	21.6	17.9	1.5	1.4	2.3
	8	5.9	9.1	11.6	1.2	1.7	1.2
29/05/2015	2	2.6	14.3	18.7	0.0	0.0	0.0
	4	12.3	16.7	9.2	0.3	0.0	0.5
	6	9.5	17.2	13.8	0.3	0.0	0.5
	8	0.0	3.5	7.5	0.3	0.1	0.4
1/06/2015	2	0.4	9.3	12.0	0.0	0.0	0.0
	4	6.6	9.9	4.5	0.5	0.0	1.3
	6	4.6	10.0	8.0	0.4	0.0	0.4
	8	0.6	0.6	3.2	0.1	0.0	0.8
3/06/2015	2	0.0	4.9	7.2	0.0	0.1	0.0
	4	1.3	5.9	1.0	0.8	0.0	0.6
	6	1.2	4.6	1.8	0.8	0.0	0.5
	8	0.0	0.0	0.9	1.7	0.7	1.0
5/06/2015	2	0.0	1.8	1.7	0.0	0.0	0.0
	4	1.0	1.2	0.9	0.4	0.0	0.1
	6	1.0	0.9	1.1	0.4	0.0	0.1
	8	0.0	0.0	0.9	1.2	0.3	0.1
8/06/2015	2	32.7	31.0	30.6	9.5	9.5	9.5
	4	29.6	29.0	30.8	9.5	9.5	9.5
	6	30.7	30.3	29.2	9.5	9.5	9.5
	8	31.3	30.1	29.6	9.5	9.5	9.5
10/06/2015	2	24.9	23.2	25.5	4.7	4.4	3.8
	4	18.7	17.4	18.3	5.3	4.7	5.1
	6	22.0	21.4	21.5	5.6	3.1	4.0
	8	21.6	18.0	13.4	4.2	4.5	4.5
12/06/2015	2	17.4	20.1	21.7	0.6	0.4	0.7
	4	17.3	13.8	14.4	0.2	0.0	0.2
	6	18.5	16.5	15.4	0.0	0.0	0.0
	8	16.2	13.0	8.8	0.1	0.3	0.0
15/06/2015	2	7.0	10.4	9.4	0.1	0.3	0.6

	4	6.7	0.2	2.3	0.2	0.0	0.1
	6	4.7	6.4	3.8	0.1	0.0	0.0
	8	3.5	0.5	0.0	0.0	0.2	0.0
17/06/2015	2	2.0	5.2	4.2	0.0	0.2	0.2
	4	1.0	0.0	0.5	0.1	0.0	0.0
	6	0.6	1.5	1.1	0.3	0.0	0.0
	8	1.2	0.0	0.0	0.0	0.0	0.0

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